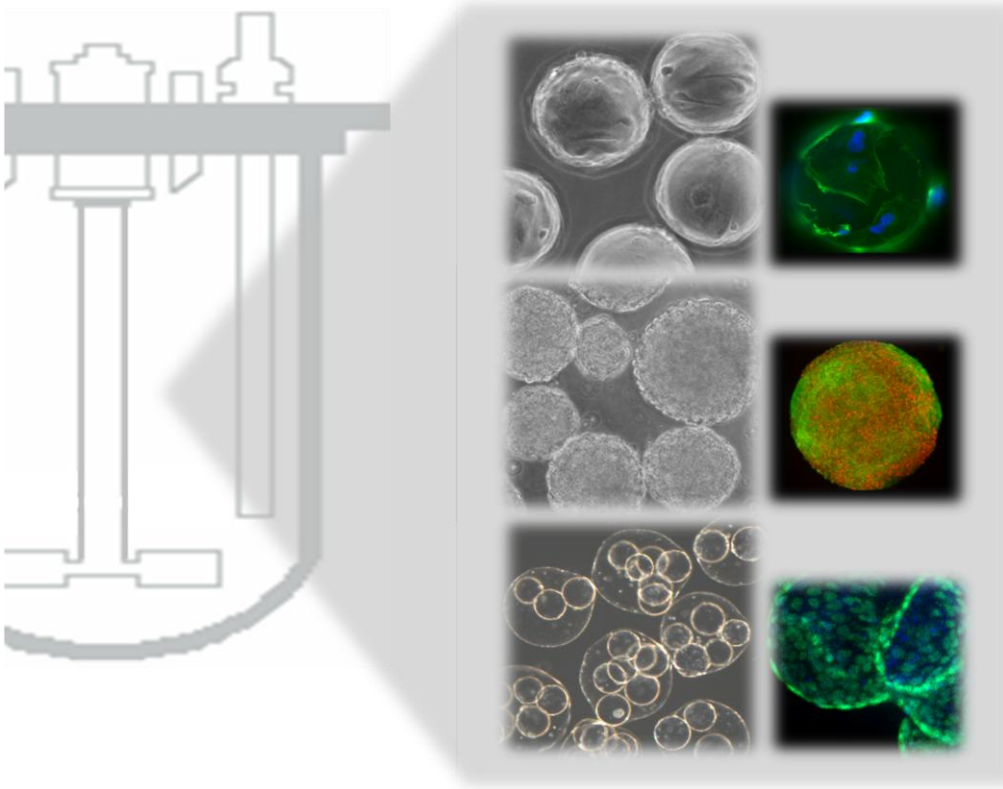
Process Engineering of Stem Cells for Clinical Application



Maria Margarida de Carvalho Negrão Serra

Dissertation presented to obtain a Ph.D degree in Engineering and Technology Sciences, Biomedical Engineering at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa

Oeiras, February 2011

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Process Engineering of Stem Cells for Clinical Application

by Maria Margarida Serra

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Front cover: Composite image of the main stem cell models and 3-D culture strategies used for the development of novel stem cell bioprocesses; phase contrast and immunofluorescence microscopy images of rPSCs immobilized on microcarriers, NT2 cell aggregates and alginate microencapsulated hESCs immobilized on microcarriers; immunofluorescence microscopy images of rPSCs labelled for nestin (green), NT2 neurosphere stained with nestin (red) and β-tubulin-III protein (green), hESCs labelled for oct-4 (green), nuclei were stained with dapi (blue).

Back cover: Neuronal differentiation of NT2 cells. Immunofluorescence microscopy images of NT2 cultures composed by non-neuronal cells (stained with nestin, red) and neurons (labelled with β-tubulin-III protein, green), nuclei were stained with dapi (blue); phase contrast image of a pure population of neurons.
By Margarida Serra and Teresa Serra

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Foreword

This thesis dissertation represents four years of research undertaken at the Animal Cell Technology Unit of the *Instituto de Tecnologia Química e Biológica* from the *Universidade Nova de Lisboa*/*Instituto de Biologia Experimental e Tecnológica* under the supervision of Dr. Paula Alves.

This thesis intends to explore efficient and scalable bioprocesses for expansion and neuronal differentiation of stem cells in order to ensure the robust production of challenging cell-based products, namely human embryonic stem cells, for clinical application.

À memória dos meus avós Clara e Camilo

Aos meus pais

À Teresa

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ABSTRACT

Over the last decade, human embryonic stem cells (hESCs) have garnered a lot of attention owing to their inherent self-renewal ability and pluripotency. These characteristics have opened opportunities for potential stem cell-based regenerative medicines, for development of drug discovery platforms and as unique *in vitro* models for the study of early human development.

With “large-scale” applications of hESCs in the horizon, the establishment of scalable and well defined culture methods that must preserve their proliferation capacity and differentiation potential is still a challenge. Currently, 2-D culture systems are well established for routine hESC cultivation. However, the inherent variability, lack of environment control and the low productions yields associated with these 2-D culturing approaches are the main drawbacks limiting their use in clinical or industrial scale.

The main focus of this thesis was the development of robust and scalable systems for the efficient production of cell-based products, capable of generating relevant numbers of well characterized cells for therapeutic and/or pharmacological applications. More specifically, novel culture strategies were explored aiming to enhance stem cell expansion and/or neuronal differentiation. On a first approach, two distinct stem cell lines were used, namely adult and teratocarcinoma stem cells, as both share important characteristics and similar bioprocessing challenges with hESCs. The preliminary knowledge gained with these stem cell systems contributed to cope with the complexity of hESC culture and fulfill the final goal of this thesis which was the implementation of robust bioprocesses for the production of pluripotent hESCs. To achieve these aims, an integrated approach was developed by combining different 3-D culturing strategies (such as cell aggregates and cell immobilized on

microcarriers) with stirred tank bioreactor technology and by addressing critical bioprocess variables.

In **Chapter 1** the recent advances in stem cell bioprocessing are reviewed, with particular relevance given to specific environmental factors impacting on stem cell fate decisions and culture outcome. A special focus is given to the current drawbacks of standard protocols for hESC cultivation and the potential of novel culture strategies and bioreactor systems to overcome them.

In **Chapter 2**, a scalable and controlled strategy was developed for the expansion of undifferentiated rat pancreatic stem cells (rPSCs) which are anchorage-dependent cells that present high proliferation capacity and differentiation potential. This was done by combining microcarrier technology with environmentally controlled stirred tank bioreactors. The use of microcarrier supports overcame the main drawbacks of aggregate culture namely by avoiding the cell clumping that prevented pluriferation. Although the two microcarriers tested were suitable for PSC culture, Cytodex 3 provided a better matrix to promote cell attachment and growth. At the end, the controlled bioprocess allows the efficient expansion of rPSCs, without compromising stem cell characteristics and differentiation potential, representing an efficient starting point towards the development of novel protocols for other stem cell lines including hESCs.

The main focus of **Chapters 3** and **4** was the development of a robust platform for the production of human neurons derived from stem cells. The rationale behind the selection of the teratocarcinoma stem cell line (NT2 cell) was based, not only on the important characteristics that these cells share with hESCs (expression of stem cell markers, high self-renewal ability and pluripotency), but also because they are a valuable model for human neuronal differentiation *in vitro;* the neurons derived

from this cell line, NT2-N, have been used as a promising biological source both for cell therapy and for drug screening investigations. The cultivation of NT2 cells as 3-D cell aggregates (“neurospheres”) in stirred tank bioreactors was the strategy adopted aiming to accomplish three main objectives: i) up-scale ii) accelerate and iii) enhance neuronal differentiation of stem cells.

Chapter 3 describes in detail the three-step protocol developed for NT2 differentiation. It was shown that, both cell-cell interactions and retinoic acid treatment presented in the 3-D neurosphere system contributed to a more efficient (4-fold) and rapid (approximately 50%) neuronal differentiation process than in the conventional cell monolayer cultures. Efforts were also directed in the optimization of cell harvesting procedures. The highest percentage of recovered neurons was achieved when intact neurospheres were transferred directly to treated surfaces, indicating that both 3-D neurosphere dynamics and the extracellular matrix surfaces are sufficient to provide an optimal system for the harvesting of NT2-N neurons.

The expansion step of NT2 aggregates was further investigated in **Chapter 4**. Different bioprocess variables were tested and the best compromise was obtained using an inoculum concentration of $4x10^5$ cell/mL and the media exchange operation mode, ensuring the fast production of high cell numbers without compromising their phenotype and differentiation potential. By incorporating both expansion and differentiation steps in an integrated bioprocess, the strategy allows for obtaining well differentiated neurons after 2 weeks of differentiation, as well as higher yields of neurons for a later culture time (10-fold improvement when compared to static culture protocols). Importantly, the bioprocess was reproduced and validated in controlled stirred tank

bioreactors, conferring process automation, scalability and reproducibility, important requirements in stem cell bioprocessing.

In **Chapters 5 and 6**, we step into the complexity of hESC cultivation. The potential of bioreactor technology was explored **in Chapter 5** to improve the expansion of pluripotent hESC on microcarriers. The importance of controlling dissolved oxygen at 30% air saturation and the impact of incorporating an automated continuous perfusion system on cell growth and metabolism were discussed, demonstrating to be critical for the production of relevant cell numbers without compromising their pluripotency. At the end an improvement of 12-fold in the final cell yield was obtained when compared to static 2-D cultures, yielding almost 7×10^8 of pluripotent hESCs *per* 300mL bioreactor run.

Cell microencapsulation in alginate was investigated in **Chapter 6** as the main strategy to improve further hESC expansion and facilitate bioprocess integration with cryopreservation protocols. For this purpose three different 3-D culture strategies were evaluated and compared: microencapsulation of hESCs as single cells, aggregates and immobilized on microcarriers. The combination of cell microencapsulation and microcarrier technology resulted in an optimum protocol for the production and storage of pluripotent hESCs. This strategy ensures high expansion ratios (approximately 19-fold increase in cell concentration) and high cell recovery yields after cryopreservation. hESCs-microcapsules were cultured in stirred tank bioreactors and, after expansion, cryopreserved in cryovials, aiming to implement a scalable and straightforward integrated bioprocess.

Chapter 7 consists of a general discussion, where main achievements and conclusions of the work are presented and future perspectives outlined.

This thesis contributes substantially to the establishment of effective methodologies for the production of challenging cell-based products such as hESCs. We believe that the 3-D culture strategies developed herein will provide a new way to streamline robust protocols for stem cell expansion and directed differentiation and potentiate the translation of stem cells and their derivatives towards a broad spectrum of applications in regenerative medicine, tissue engineering and *in vitro* toxicology.

RESUMO

Durante a última década, as células estaminais embrionárias humanas (hESCs) despertaram muita atenção devido à sua capacidade de auto-renovação e pluripotência. Estas propriedades conferem às hESCs uma enorme aplicabilidade em medicina regenerativa, no rastreio de novos fármacos e em investigação científica por constituírem modelos celulares únicos para o estudo e compreensão dos processos de desenvolvimento embrionário inicial.

Contudo, a aplicação crescente das hESCs requer ainda o desenvolvimento de metodologias de cultura bem definidas e reproduzíveis em maior escala, que garantam a manutenção das propriedades de auto-renovação e diferenciação das células após o processo. Os métodos mais correntemente utilizados para cultura de hESCs são as monocamadas bi-dimensionais (2-D). No entanto, a baixa reprodutibilidade, a falta de controlo ambiental e os baixos rendimentos celulares associados a estas abordagens de cultura 2-D limitam a utilização destes sistemas numa escala clínica ou industrial.

O principal objectivo desta tese consistiu em estabelecer sistemas de cultura para a produção eficiente de células estaminais, que permitam o redimensionamento e robustez do processo e que sejam capazes de gerar elevados números de células bem caracterizadas para terapia e/ou para aplicações farmacêuticas. Especificamente, foram exploradas estratégias de cultura de células de forma a melhorar os processos de expansão e de diferenciação neuronal. Numa primeira abordagem, foram usadas duas linhas de células estaminais, nomeadamente células estaminais adultas e células estaminais de um teratocarcinoma, que apresentam características biológicas e de cultura muito semelhantes às hESCs.

O conhecimento preliminar adquirido com esses sistemas celulares permitiu compreender a complexidade da cultura de hESC e implementar bioprocessos robustos para a produção de hESCs pluripotentes. Foi desenvolvida uma abordagem integrada através da combinação de diferentes estratégias de cultura tri-dimensionais (3-D) (tais como agregados celulares e células imobilizadas em microsuportes) com a tecnologia de bioreactores de tanque agitado, e através da manipulação de diversas variáveis críticas ao bioprocesso.

No **Capítulo 1**, é apresentada uma introdução geral ao tema de bioprocessamento de células estaminais, incidindo no impacto de determinados factores ambientais na cultura de células estaminais. É dada particular relevância ao estado da arte relativamente às limitações dos sistemas tradicionais de cultura em 2-D, bem como ao potencial de novas estratégias de cultura 3-D e à tecnologia de biorreatores para ultrapassar essas limitações.

No **Capítulo 2**, foi desenvolvida uma estratégia escalonável e robusta para a expansão de células estaminais pancreáticas de rato (rPSCs), que são células que apresentam uma grande capacidade de proliferação e diferenciação e cuja cultura é dependente de uma matriz/suporte. Para tal, as rPSCs foram cultivadas em microsuportes num ambiente controlado, usando bioreactores de tanque agitado. O uso de microsuportes superou as principais desvantagens da cultura de agregados celulares, onde as células se agruparam e não proliferaram. Embora os dois tipos de microsuportes testados tenham sido eficientes na cultura de rPSC, os microsuportes Cytodex 3 proporcionaram melhor aderência e crescimento às células. No final, o bioprocesso controlado permitiu a expansão eficiente de rPSCs, sem comprometer as características biológicas das células nem o seu potencial de diferenciação, o que representa um ponto de partida relevante para a

implementação de protocolos promissores para outras linhas de células estaminais, incluindo hESCs.

O principal objectivo dos **Capítulos 3** e **4** foi o desenvolvimento de uma plataforma robusta para produção de neurónios humanos derivados de células estaminais. O racional por detrás da selecção da linha celular de células estaminais de um teratocarcinoma (NT2) baseou-se, não só pelo facto destas células apresentarem características importantes e semelhantes às hESCs (expressão de marcadores de células estaminais, capacidade de auto-renovação e pluripotência), mas também por constituírem um bom modelo celular para a diferenciação neuronal *in vitro*; os neurónios derivados desta linha celular, NT2-N, têm sido usados em ensaios de terapia celular e no desenvolvimento de novos fármacos. Nestes capítulos, as células NT2 foram cultivadas como agregados 3-D de células ("neurosferas") em biorreatores de tanque agitado com o objectivo de: i) aumentar a escala ii) acelerar e iii) melhorar o processo de diferenciação neuronal de células estaminais.

O **Capítulo 3** descreve em pormenor o protocolo 3-D estabelecido para a diferenciação neuronal de células NT2. Verifiou-se que, as interacções célula-célula e o tratamento com ácido retinóico presentes no sistema 3-D contribuíram para um processo de diferenciação mais eficiente (4 vezes) e mais rápido (cerca de 50%) comparativamente com as culturas convencionais em sistema estático. Neste capítulo foram ainda optimizados métodos de recolha e selecção de neurónios com o intuito de obter elevadas percentagens de recuperação. O método mais eficaz correspondeu ao processo de transferência directa das neurosferas intactas para superfícies tratadas com proteínas da matriz extracelular, indicando que, tanto a dinâmica celular das neurosferas como as propriedades da matriz são suficientes para fornecer um sistema ideal para recolha e selecção de neurónios NT2-N.

No **Capítulo 4** foi investigada a etapa de expansão de células NT2 na forma de agregados. Diferentes variáveis de processo foram estudas e o melhor compromisso foi obtido utilizando uma concentração de inóculo de $4x10^5$ célula/mL e a mudança de meio como modo de operação da cultura. Estas condições garantiram a produção rápida de números elevados de células sem comprometer o seu fenótipo e potencial de diferenciação. Ao integrar as etapas de expansão e diferenciação, o bioprocesso desenvolvido permitiu obter neurónios diferenciados em apenas duas semanas de diferenciação. Para além disso, no final da terceira semana, o número de neurónios produzidos foi significativamente superior quando comparado com o protocolo de cultura em sistema estático (aumento de 10 vezes da eficiência de diferenciação). No fim, o bioprocesso foi reproduzido e validado em bioreactores de tanque agitado e em ambiente controlado, conferindo ao processo automatização, escalabilidade e reprodutibilidade, requisitos importantes no bioprocessamento de células estaminais.

Os **Capítulos 5** e **6**, descrevem já um nível de complexidade superior, utilizando culturas de hESCs. O potencial da tecnologia de bioreactores, adquirido nos Capítulos 3 e 4, foi explorado no **Capítulo 5** com o objectivo de melhorar a expansão de hESCs pluripotentes em microsuportes. A importância do controlo de oxigénio dissolvido (30% de ar saturado) e o impacto da perfusão contínua da cultura no crescimento celular e no metabolismo foram estudados, e demonstraram ser parâmetros fundamentais para a produção de hESCs em elevadas quantidades sem comprometer a sua pluripotência. No final, o rendimento celular foi melhorado 12 vezes relativamente aos métodos de culturas 2-D em sistema estático, garantindo cerca de 7×10^8 hESCs pluripotentes por cada ensaio em biorreactor de 300mL.

No **Capítulo 6** foi explorada a tecnologia de microencapsulação de células em alginato com o objectivo de melhorar o processo de expansão de hESCs e desenvolver um bioprocesso integrado com protocolos de criopreservação. Para este efeito, foram avaliadas e comparadas diferentes estratégias de cultura 3-D, nomeadamente a microencapsulação de hESCs como: células individualizadas, agregados de células e células imobilizadas em microsuportes. A combinação da microencapsulação de células com a tecnologia microsuportes resultou num protocolo eficiente para a produção e armazenamento de hESCs pluripotentes. Esta estratégia garantiu rendimentos de expansão celular elevados (aumento de cerca de 19 vezes na concentração de células) e percentagens de viabilidade celular altas após a criopreservação. As culturas de hESCs microencapsuladas foram cultivadas em biorreactores de tanque agitado e, após a expansão, criopreservadas em criotubos, visando a implementação de um bioprocesso simples integrado e possível de aumento de escala.

O **Capítulo 7** consiste numa discussão geral, onde são apresentadas os principais resultados, conclusões e as perspectivas futuras do trabalho.

Em resumo, esta tese contribui significativamente para o estabelecimento de metodologias eficazes para a produção de sistemas celulares complexos, tais como as hESCs. As estratégias inovadoras de cultura 3-D aqui apresentadas poderão acelerar o desenvolvimento de protocolos robustos para a expansão e diferenciação dirigida de células estaminais e consequentemente potenciar a transição destas células e seus derivados para um amplo espectro de aplicações em medicina regenerativa, engenharia de tecidos e no desenvolvimento de novos fármacos.

THESIS PUBLICATIONS

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ABBREVIATIONS

Abbreviation	Full form
µ	apparent growth rate
2-D	two-dimensional
3-D	three-dimensional
ANOVA	analysis of variance (statistics)
AP	alkaline phosphatase
AraC	cytosine arabinoside
ASCs	adult stem cell
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth facto
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
DMEM-HG	Dulbecco's modified Eagle medium with high glucose concentration
EBs	embryoid body
EC	embryonal carcinoma
ECM	extracellular matrix
ELISA	enzyme linked immuno sorbent assay
ESC	embryonic stem cell
FBS	foetal bovine serum
FDA	Food and Drug Administration
FI	fold increase in cell expansion
FOX A2	forkheadbox A2
FSG	fish skin gelatin
Fudr	fluorodeoxyuridine
GFAP	glial fibrillary acidic protein
GLC	glucose
GLN	glutamine
GMP	good manufacturing practices
GRNOPC1	geron’s oligodendrocyte progenitor cells derived from hESCs
HARV	high aspect rotating vessel
hESC	human embryonic stem cell

hESCellect™	antibody with high specificity against surface epitopes in undifferentiated hESCs
hFF	human foreskin fibroblasts
hFFCellect™	antibody with high specificity against surface epitopes in undifferentiated hFFs
HGF	hepatocyte growth factors
ICM	inner cell mass
IgG	immunoglobulin G
IgM	immunoglobulin M
IPATIMUP	Instituto de Patologia e Imunologia Molecular da Universidade do Porto
iPSC	induced pluripotent stem cell
k_d	apparent death rate
Ki67	protein that is encoded by the *MKI67* gene (antigen identified by monoclonal antibody Ki-67); marker to determine the growth fraction of a given cell population
KO-DMEM	knock out Dulbecco's modified Eagle medium
KO-SR	knock out serum replacement
LAC	Lactate
LDH	lactate dehydrogenase
MAP2a&b	microtubule associated protein-2
mESC	mouse embryonic stem cell
MG	Matrigel
MSC	mesenchymal stem cell
NF200	heavy chain neurofilament,
NF-L	light chain neurofilament
NH4+	Ammonia
NT2	human embryonal carcinoma stem cell line NTera-2/cl.D1
NT2-N	neurons derived from NT2 cells
O4	oligodendrocyte marker O4
P/S	penicillin-streptomycin
PAM	pharmacologically active microcarriers
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDL	poly-D-Lysine
PFA	Paraformaldehyde
PLGA	poly(D,L-lactic-*co*-glycolic acid)

pO_2	dissolved oxygen
q_{GLC}	specific rate of glucocose consumption
q_{LAC}	specific rate of lactate production
q_{LDH}	specific rate of LDH release
qRT-PCR	quantitative real time polymerase chain reaction
RA	retinoic acid
RCC	rotary cell culture
RNA	ribonucleic acid
rPSCs	rat pancreatic stem cells
SCED™461	hESC line; feeder-cell based culture system developed for the easy propagation of hES cells by enzymatic digestion (SCED- single cell enzymatic dissociation)
SSEA-1	stage specific embryonic antigen-1
SSEA-4	stage specific embryonic antigen-4
STLV	slow turning lateral vessel
t_d	doubling time
TGFβ	transforming growth factor beta
TRA-1-60	tumor related antigen-1-60
TRA-1-60	tumor related antigen-1-60
TRAP	telomeric repeat amplification protocol
TX-100	triton X-100
UK	United Kingdom
Urd	uridine
VEGF	vascular endothelial growth factor
Xmax	maximum cell concentration
$Y_{LAC/GLC}$	yields of lactate production from glucose consumption
α-SMA	α-smooth muscle actin
β-TubIII	anti-tubulin beta III isoform

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CHAPTER 1

INTRODUCTION

This chapter was based on the following manuscript:

Serra, M., Brito, C. and Alves, P.M., 2010. Bioengineering strategies for stem cell expansion and differentiation. Canal Bioquímica 7, 30-38.

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1. INTRODUCTION

Human embryonic stem cells (hESCs) constitute an exciting emerging field. The inherent capacity of these cells to grow indefinitely (self-renewal) and their ability to differentiate into all mature cells of the human body (pluripotency), have made them an extremely attractive tool for regenerative medicine and tissue engineering (Nirmalanandhan and Sittampalam, 2009). Indeed, for many years, they are considered the great promise for treating degenerative disorders such as Parkinson diseases, type I diabetes and heart failure, hoped to provide a new source of neurons, insulin producing cells or cardiomyocytes to replace the degenerating tissues and/or impaired cells.

The first clinical trial with hESCs was approved by the US Food and Drug Administration (FDA) in January 2009 but only initiated in October 2010. The goal of this first study is to assess the safety and tolerability of oligodendrocyte progenitor cells derived from hESCs (GRNOPC1, Geron Comp.) in patients with neurologically complete spinal cord injuries, i.e. patients with complete loss of locomotor and sensory activity below the site of injury. The second endpoint is efficacy; it will use similar testing for evidence of any return of sensory function or lower extremity locomotion for one year after injection of GRNOPC1. This clinical trial holds high expectations as in previous experiments with rats these cells revealed to be safe and efficient in restoring some function (Zhang et al., 2006).

Recently, another hESC clinical trial was approved (November 2010) by the US FDA. Advanced Cell Technology, Inc. announced a Phase I/II multicenter clinical trial using retinal cells derived from hESCs to treat patients with Stargardt's Macular Dystrophy (SMD), one of the most common forms of juvenile macular degeneration in the world.

Today an increasing number of biotechnology industries have focused their interest on the development of both adult and embryonic stem cells as therapies; some examples are listed in Table 1.1.

In addition to clinical applications, hESCs have enormous prospective for the development of novel technologies in drug screening (Davila *et al.*, 2004; Ebert and Svendsen, 2010; Jensen *et al.*, 2009). In fact, given the high costs spent by pharmaceutical R&D to bring a new drug to market, there is ongoing effort to introduce new cell models which are practicable (robust, reproducible, etc.) and have improved throughput and predictivity. hESCs and their derivatives have all the potential to be used here as "bio-tools", also contributing to the reduction of animal experimentation (Davila et al., 2004; Jensen et al., 2009). For example, pure cultures of hepatocytes, cardiomyocytes and neuronal cells derived from hESCs would provide robust cell-based *in vitro* assays for toxicity measurements and for drugs being development for cardiovascular or neurodegenerative disorders, respectively.

hESCs are also valuable models for scientific research. They can lead to a better understanding of the basic biology of the human body, embryonic development, pathogenesis of congenital defects and cancer formation (Bongso et al., 2008). In fact, it is possible to derive disease-specific hESCs from embryos with diagnosed mutations by preimplantation genetic diagnosis (Galat et al., 2010). As an example, hESC lines derived from embryos with Fanconi anemia-A mutation and fragile X mutation have already been established (Galat et al., 2010). These hESC lines will provide in *vitro* models for study the phenotype of these mutations, allowing the faster identification of new treatments for these diseases.

Table 1.1. Summary of some biotechnology industries that have focused their research on the development of both adult and embryonic stem cells as therapies. The main technologies developed and ongoing actions are presented.

Company	Technology	Indications	Action and Clinical Status
Aastrom Biosciences Inc *(Minnesota,USA)*	Autologous therapy (patient´s own cells) Tissue repair cells	Limb ischemia, bone, cardiac regeneration	Phase II, III, I clinical trial
Advanced Cell Technology Inc *(California, USA)*	Multiple technologies based on ESC	Heart failure, macular degeneration , vascular ischemia	Received U.S. FDA approval for use retinal stem cells to treat Stargardt's Macular Dystrophy Phase I/II
Aldagen Inc *(North Carolina, USA)*	ALD-201	Heart failure	Phase I clinical trial
AmStem, Histostem *(California, USA)*	Human umbilical cord blood stem cells	Buerger´s disease Hair loss treatment	Phase I and II clinical trial completed Received Korea FDS approval Phase II and III clinical trials
Athersys (ATHX) *(Ohio, USA)*	MultiStem	Myocardial infarction, bone marrow transplantation	Phase I clinical trial
Celgene Corp *(New Jersey, USA)*	Blood cancer treatments		Phase I clinical trials
Cytori Therapeutics Inc *(California, USA)*	Adipose tissue derived stem cells	Tissue regeneration after breast surgery, cardiac ischemia, hear attack	In planning, pilot study Its StemSource product line is sold globally for cell banking and research applications.
Geron Corp. *(California, USA)*	hESCs derived oligodendrocytes	Spinal cord injury	Received U.S. FDA approval Phase I
Neuralstem Inc *(Maryland, USA)*	Neural Stem cells	CNS injury (chronic spinal cord injury, amyotrophic lateral sclerosis)	Recently filed a new drug application with the FDA to begin a Phase I safety clinical trial for chronic spinal cord injury
NovoCell *(California, USA)*	Stem cell engineering, cell encapsulation	Type I diabetes	Proof of concept phase I/II
Osiris Therapeutics Inc. *(Maryland, USA)*	Autologous therapy (patient´s own cells taken from the bone marrow)	Crohn's disease	Phase III clinical trial
ReNeuron Group Plc *(Guildford,UK)*	REN009 stem cell therapy	peripheral arterial disease in diabetes	Starting trials in 2011.
StemCells Inc. *(California, USA)*	Human neural stem cells (HuCNS-SC® product)	Batten disease	Starting human trials in 2011

	Technology
Apceth *(München,Germany)*	Development of GMP-grade protocols for mesenchymal stem cell production and gene transfer for cancer therapy applications
International Stem Cell Corporation *(California, USA)*	Establishment of human stem cells via parthenogenesis (hpSC lines) to provide potential products as alternatives to ESCs. ISCO plans to create a bank of these hpSC lines (UniStemCell™)
Lonza Bioscience *(Basel, Switzerland)*	Establishment of Poietics® Human Adipose-Derived Stem Cells for use in adult stem cell research
Thermogenesis Corp *(California, USA)*	Supply of products and services that process and store adult stem cells

2. TRANSFERRING STEM CELLS TO THE CLINIC: WHAT IS NEEDED?

The successful translation of stem cells to the clinical and/or industrial fields will require contributions from fundamental research (from the developmental biology to the "omics" technologies and advances in immunology) and from existing industrial practice (biologics), especially on automation, quality assurance and regulation.

Attention is shifting also to the development of bioprocesses to produce hESCs or their derivatives in high purity, consistent quality and relevant quantity.

2.1. Purity

The tumorigenic potential of pluripotent stem cells is one of the important hurdles in the safety utilization of these cells. At present, protocols for the directed differentiation of stem cells are generally inefficient, resulting in low differentiated cell yields and contamination by other cell types. Of greater concern is the persistence of undifferentiated stem cells and the possibility of these cells form malignant tumors when transplanted in the host (Fujikawa et al., 2005). Therefore the use of efficient methods for differentiation and selection of pure populations of specialized cells will be essential before these cells being used clinically (Brignier and Gewirtz, 2010).

2.2. Quality

To develop cell-based products with clinical quality, procedures (e.g. isolation, propagation, differentiation, cryopreservation) and compounds (e.g. matrices, culture and cryopreservation media, supplements) have to minutely follow the FDA regulations (Holm et al., 2010). Importantly, cell phenotype and function should be characterized and evaluated during

culture. Undifferentiated stem cells have to maintain their pluripotency and genetic and epigenetic stability after expansion while stem cell derivatives must express markers of the specific cell lineage and be fully functional after differentiation.

2.3. Quantity

Another important challenge is to achieve sufficient numbers of stem cell for an effective therapy. In general, these numbers fall in the range of millions to a few billion. For example, in Geron´s first clinical trial, patients will be injected in the spinal cord with small doses of GRNOPC1 ($2x10^6$ cells, www.geron.com), but for the replacement of damage cardiac tissue after myocardial infarction $1\text{-}2x10^9$ cardiomyocytes are required (Jing *et al.*, 2008). To achieve these high cell numbers robust, affordable and scalable bioprocesses need to be developed.

3. STEM CELL BIOPROCESSING

The successful production of stem cell-based products relies on robust bioprocesses that should be designed following pertinent principles (Figure 1.1, Placzek et al., 2009).

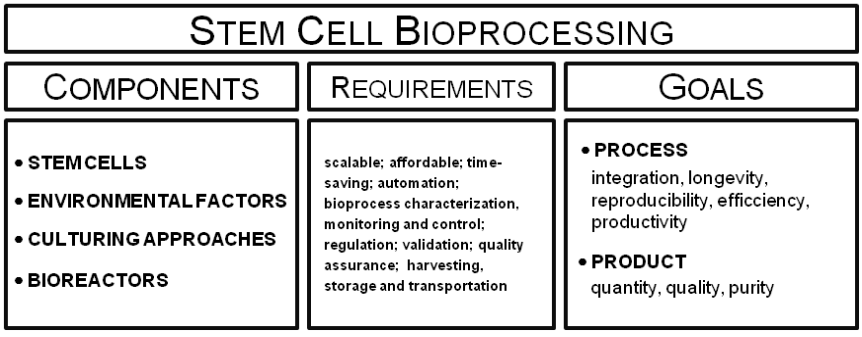

Figure 1.1. Design principles for stem cell bioprocessing (Adapted from Placzek et al. 2009).

Herein, the cell source and the signals that govern stem cell fate decisions are essential bioprocessing components. Next, the integration of a controlled culturing strategy for 3-D cell organization via cell self-assembly, cell immobilization to biomaterials/supports with a bioreactor-based system where the necessary conditions for cells to guide their fate are “perfectly tuned”, is a key factor to move stem cells from lab scale to clinical trials and large scale industrial applications. In this chapter, the importance of these process components on the design of stem cell bioprocesses will be presented, highlighting the main requirements needed to fulfil the end product’s purity, quality and quantity.

3.1. Stem cell sources

There are several classes of stem cells including embryonic and adult stem cells, and the new type of induced stem cells, each one presenting its own benefits, limitations and challenges in bioprocess development (Figure 1.2). All of them share as common features the ability to proliferate indefinitely (unlimited self-renewal capacity) and vary in their differentiation potential.

hESCs are isolated from the inner cell mass (ICM) of blastocysts at day five of embryonic development. The first reports of hESCs were published in 1984 (SB Fishel et al., 1984) and 1994 (Bongso et al., 1994), but it was only in 1998 that Thomson and co-workers described the isolation of hESCs and the establishment of the firsts permanent and characterized hESC lines for research (Thomson et al., 1998). Today, more than 1000 hESC lines are reported in the literature (Löser et al., 2010). Some of these cell lines are well characterized and organized in international stem cell banks, for example, hESCreg (www.hescreg.eu), UK stem cell bank (www.ukstemcellbank.org.uk), and National stem cell bank (www.nationalstemcellbank.org).

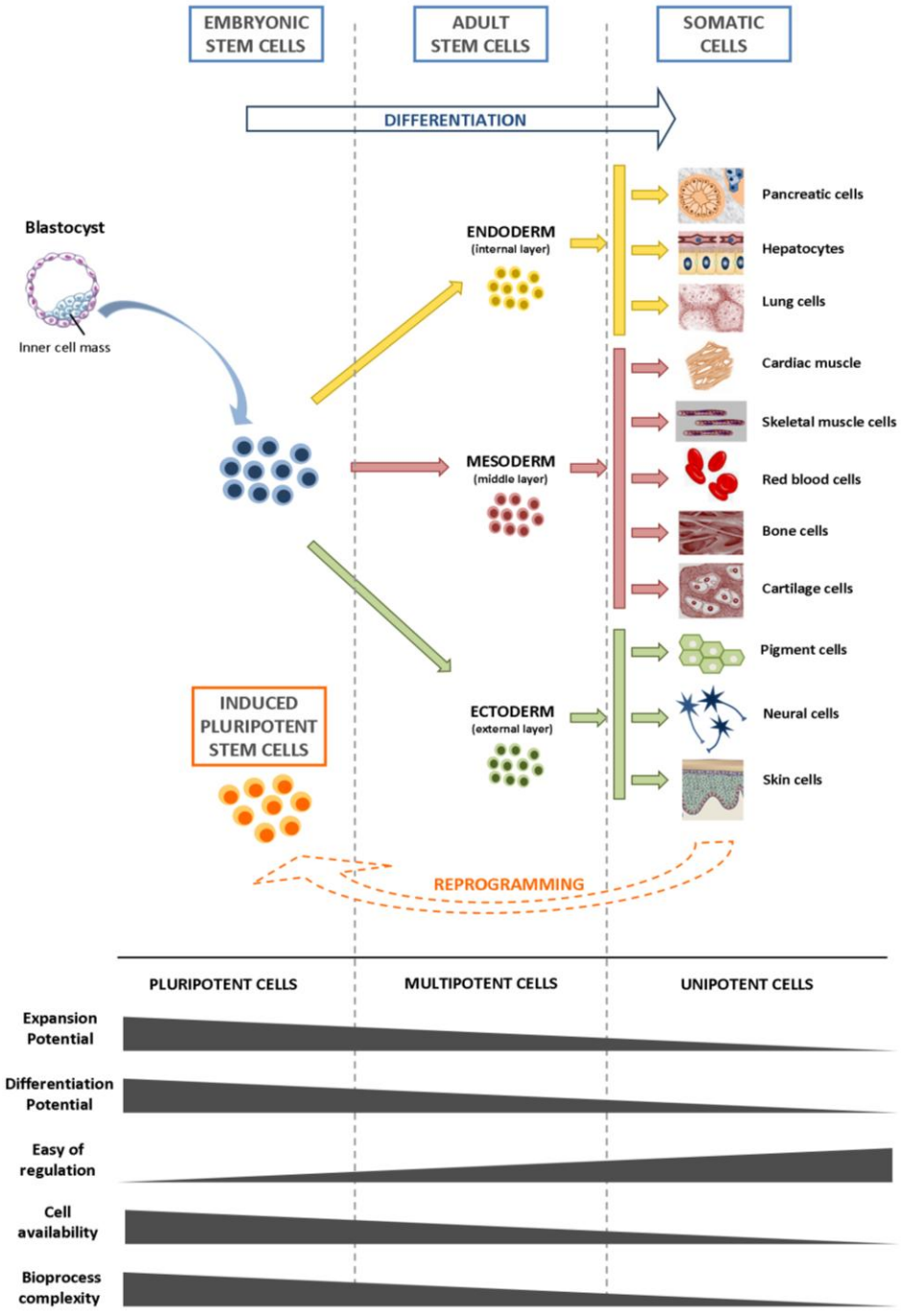

Figure 1.2. Stem cell sources and characteristics. (Adapted from (Placzek *et al.*, 2009)).

Substantial efforts have been made towards the identification of phenotypic/genomic markers to characterize, validate and distinguish hESCs from other cell types. hESC lines can be identify by the presence of surface marker antigens (Tra series, SSEA series, GCT series, HLA, and CD markers) and transcriptional factors (Oct4, Nanog), by the chromossomal stability with serial culture, alkaline phosphatase positiveness and high telomerase activity (Allegrucci and Young, 2007; Bongso et al., 2008). As described above, these cells present a high proliferation capacity and are pluripotent, i.e., they possess the potential to differentiate into all cell types that compose an adult body, derived from the three germ layers (e.g. cardiomyocytes, neurons, pancreatic islets, hepatocytes, chondrocytes,) (Hay et al., 2007; Kroon et al., 2008; Mummery et al., 2003; Toh et al., 2009; Zhang et al., 2001). However, hESCs are still difficult to control with respect to their stem cell fate, and elicit ethical considerations, requiring the manipulation of human embryos. For clinical applications, these cells still present limitations related with immune rejection and the possibility of teratoma formation. On the other hand, adult stem cells (ASCs) do not present immunogenic complications on implantation since they can be isolated directly from the patient. ASCs exist in specific niches in the different organs (e.g. bone marrow, peripheral blood, pancreas, lung, brain, liver) (Lanza et al., 2004) contributing to the regeneration/repair of the tissue/organ where they reside. Depending on the source, ASCs can be isolated relatively easy, however they present as major limitations the difficulty in obtaining pure populations, the limited expansion capacity and the restricted differentiation potential, as they are often committed to their original cell lineage (multipotent cells).

One of the most important and promising achievements in the stem cell field was the reversion of somatic cells (e.g. fibroblasts, keratinocytes) to a

state of pluripotency using defined reprogramming strategies including overexpression of a core transcription factors known to be required for maintenance of ESC pluripotence and proliferation (Oct4, Sox2, and either c-Myc and Klf4 or Nanog and Lin28) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). The creation of these induced pluripotent stem cells (iPSCs) elicited an explosion of scientific curiosity and industrial interest. This is mainly because iPSCs are similar to ESCs (namely cell morphology, cell-surface markers, self-renewal ability, potential to differentiate *in vitro* and *in vivo* into cells derived from all three germ layers) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) and thereby could potentially replace ESCs for clinical applications, circumventing the ethical concerns regarding the use of embryos. Additionally, iPSCs present the benefit of being patient-derived cells, avoiding immune rejection in cell therapy applications. iPSC research is expanding rapidly, including modeling complex diseases *in vitro* and pursuing novel therapeuticals (Selvaraj et al., 2010). However, generation of iPSCs still suffers from low efficiency and high costs revised in (Brignier and Gewirtz, 2010). Furthermore the viral expression vectors used to obtain iPSCs (Fenno et al., 2008), the potential for insertional mutagenesis (Yamanaka and Blau, 2010) and the recent knowledge that hiPSCs expresses cancer hallmarks (Malchenko et al., 2010) have raised additional concerns regarding the safety of these cells. Currently, the possibility of reprogramming somatic cells into less immature developmental stages that could be more directly applicable to therapeutic applications is being intensely explored (Jang et al., 2010; Vierbuchen et al., 2010; Zhu et al., 2010).

3.2. Environmental factors that determine stem cell fate decisions

Stem cells develop their behaviour from cues that lie in the extracellular environment. These cues operate on different temporal and spatial scales, driving specific cellular behaviours and ultimately promoting/controlling cells' self-renewal, differentiation or apoptosis (Figure 1.3).

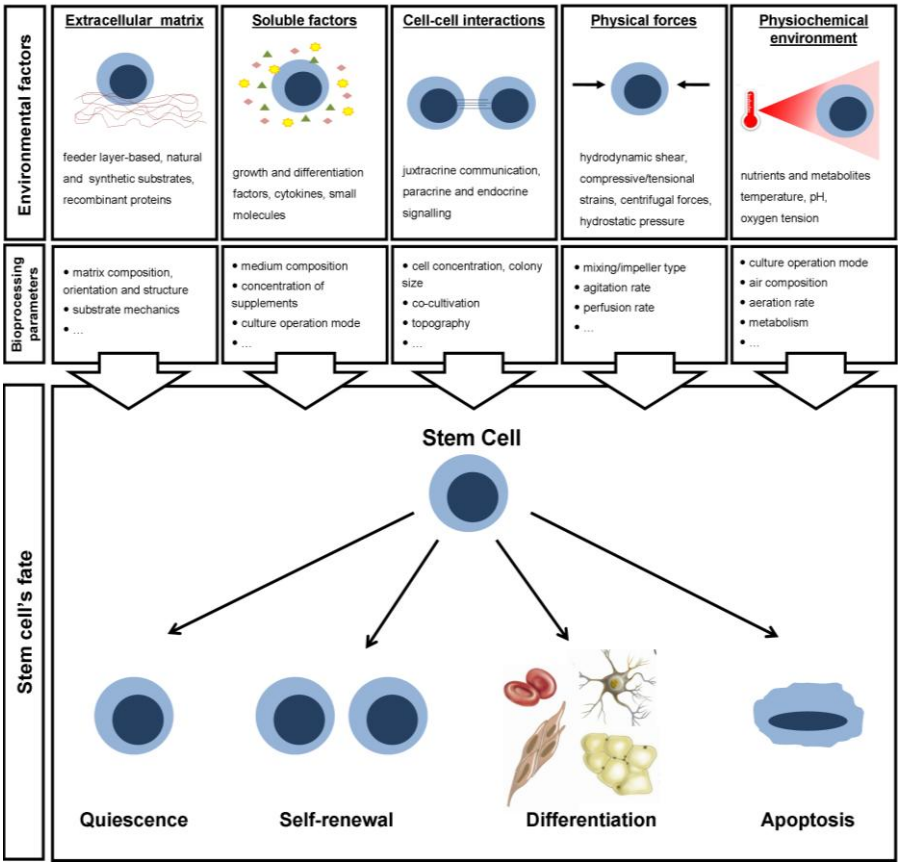

Figure 1.3. Environmental factors and bioprocessing parameters impacting stem cell fate decisions (quiescence, self-renewal, differentiation and apoptosis).

Substantial efforts have been made to identify such *stimuli*. The extracellular matrix (ECM), soluble factors, cell-cell interactions, physical forces and physiochemical factors have been suggested as the most relevant cues governing stem cell fate.

3.2.1. Extracellular matrix

Extracellular matrix (ECM) is a key component of the stem cell niche *in vivo* and can influence stem cell fate via mediating cell attachment and migration, presenting chemical and physical cues, as well as binding soluble factors. In a natural setting, this environment encloses a complex and dynamic network of proteins, polysaccharides, proteoglycans and water that provide structural and organizational guides for tissue development. The activation of these signalling pathways through the adhesion of specific components of the ECM to cells via integrins/cadherins/cell surface receptors is not trivial as it is highly dependent on the composition, orientation and structure of the ECM (Lukashev and Werb, 1998).

A wide range of animal and human-derived and recombinant protein matrices are normally used to support self-renewal or direct differentiation of hESCs (Table 1.2). hESCs are typically cultured directly of feeder cells (mouse embryonic fibroblasts, human foreskin fibroblasts) or on Matrigel, a basement membrane matrix extracted from Engellbreth-Holm-Swarm mouse tumors. However, these substrates are complex, poorly-defined and xenogenic and thus, large efforts have been done in developing defined matrixes for hESCs cultivation (Hakala et al., 2009). At least one cell subtract material composed by relatively well defined components is commercially available (CELLstart™ from Invitrogen, www.invitrogen.com).

Table 1.2. Summary of substrates used for propagation and/or differentiation of hESCs. (Adapted from (Abraham et al., 2009))

Substrate	Expansion (timeline)	Differentiation (cell lineage)	Ref.
Feeder layer based			
Human foreskin fibroblasts	>70 passages	-	(Amit et al., 2003; Choo et al., 2004; Hovatta et al., 2003)
Fetal skin cells	20 passages	-	(Richards et al., 2002; Richards et al., 2003)
Adult marrow cells	13 passages	-	(Cheng et al., 2003)
Human adult uterin endonmetrial cells	90 passages	-	(Lee et al., 2004)
Human placental fibroblasts	>25 passages	-	(Genbacev et al., 2005; Kim et al., 2007)
hESCs-derived fibroblasts	30-52 passages	-	(Stojkovic et al., 2005b; Wang et al., 2005)
Mouse bone marrow cell line S17	-	Hematopoietic	(Kaufman et al., 2001)
Yolk sac endothelial line C166	-	Hematopoietic	(Kaufman et al., 2001)
Human periodontal ligament fibroblasts	-	Osteogenic	(Inanc et al., 2007)
Natural substrates			
Matrigel ™	130 passages	-	(Xu et al., 2001)
Human serum	>27 passages	-	(Stojkovic et al., 2005a)
Collagen IV + vitronectin+ laminin+ fibronectin	Derivation of hESCs		(Ludwig et al., 2006)
Mouse embryonic fibroblasts ECM	>30 passages	-	(Klimanskaya et al., 2005)
Hyaluronic acid	20 days	-	(Gerecht et al., 2007a)
Collagen scaffolds	-	Hepatic	(Baharvand et al., 2006)
Alginate scaffolds	-	Hematopoietic	(Gerecht-Nir et al., 2004b)
Synthetic substrates			
Poly- (glycerolcosebacate)- acrylate	1 week	-	(Gerecht et al., 2007b)
Polyurethane microwells	>21 days	-	(Mohr et al., 2006)
Poly (N-isopropyl acrylamide-co-acrilic acid) SIPN	5 days	-	(Li et al., 2006)
Dextran-based hydrogels with immobilized RGD peptide and VEGF	-	Vascular differentiation	(Ferreira et al., 2007)
Poly (D,L-lactide) scaffolds	-	Osteogenic	(Bielby et al., 2004)
Poly (L-lactic acide) and poly(lactic-co-glycolic acid)			
+ retinoic acid	-	Neuronal	(Levenberg et al., 2003)
+ TGF-beta	-	Chondrogenic	
+ activin A and IGF	-	Pancreatic	
Poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide] PMEDSAH	25 passages	-	(Villa-Diaz et al., 2010)
High-affinity disulphide-bridgedRGD peptide, CRGDC	10 passages	-	(Kolhar et al., 2010)
Peptide-acrylate surfaces PAS	10 passages	Cardiomyocytes	(Melkoumian et al., 2010)
Recombinant proteins			
Human recombinant laminin-511	20 passages	-	(Rodin et al., 2010)

From clinical and industrial perspectives, the use of synthetic matrices may offer greater advantages in terms of reproducibility, quality control and costs (Kolhar et al., 2010; Melkoumian et al., 2010; Villa-Diaz et al., 2010). These matrices have wide diversity in properties that may be obtained and tailored with respect to mechanics, chemistry and degradation according to the case study. However, potential limitations to the use of synthetic materials include toxicity and limited repertoire of cellular interactions, unless they are modified with adhesion peptides or designed to release biological molecules.

3.2.2. Soluble factors

The outcome of stem cell culture depends also on the presence/concentration of growth/differentiation factors which provide survival, proliferation, differentiation signals to the cells. These regulatory molecules can be either added to the culture or secreted by the cells. Upon diffusion through the medium, these factors are sequestered by the ECM and bind to the cell surface receptors thus activating cellular functions. In alternative, to achieve a better control of the cellular microenvironment and ultimately enhance stem cell proliferation and/or differentiation, they can be immobilized on the surface of biomaterials (Ferreira et al., 2007).

Substantial efforts have been made to identify the factors regulating stem cell proliferation and/or differentiation. As an example, the basic fibroblast growth factor (bFGF) and several members of the transforming growth factor beta (TGFβ) superfamily of ligands have been reported as vital components for the self-renewal of hESCs (revised in Azarin and Palecek, 2010), while brain-derived neurotrophic factor (BDNF), hepatocyte growth factors (HGF) and vascular endothelial growth factor (VEGF) have been used to direct stem cell differentiation into specialized cell types (revised in Ulloa-Montoya et al., 2005). Some concerns regarding the use of growth

and differentiation factors in scalable culture systems is their high costs and low stability in medium. The engineering of more stable molecules or the development of appropriated perfusion systems would be potential strategies to reduce the concentration of these compounds without compromising the culture outcome.

In parallel, attempts have been made to minimize the use of these factors, for example, by including natural and/or synthetic small molecules that can be isolated/synthesized economically. Small molecules have been shown to target specific signal transduction pathways (e.g. Wnt, Hedgehog, retinoid, NF-κB), which either alone or in concert dictate the fate of stem cells, including the maintenance of undifferentiated phenotype (Sato et al., 2004) and pluripotency (Miyabayashi et al., 2007), improve cell viabilities (Watanabe et al., 2009) and promote differentiation of stem cells to cardiac (Tseng et al., 2006), hematopoietic (Naito et al., 2006), neuronal (Ding et al., 2003), and bone (Wu et al., 2004) cell phenotypes. With the advent of high-throughput screening technologies, small molecule libraries have been analyzed to identify molecular interactions leading to particular stem cell responses (revised in Ding and Schultz, 2004; McNeish, 2007).

3.2.3. Cell-cell interactions

Cell-to-cell communication, either *in vivo* or *in vitro*, can be established via direct contact (juxtacrine communication) or over distance via the diffusion of soluble signals secreted from closer (paracrine signalling) or distant (endocrine signalling) neighbouring cells. While juxtacrine cell-cell communication provides a persistent morphogenic cue, allowing the precise control of cellular responses, paracrine signaling is normally time-constrained. The extent of such limitation is dependent on the spatial distance between proximal population of cells. This occurs because signalling molecules may: 1) degrade very quickly, limiting their

effectiveness; 2) be taken by the cells very quickly, leaving few to travel further, thus creating a heterogeneous environment where cells are exposed to different concentration gradients; 3) have their movement hindered by the ECM. These different cell-cell interactions drive a set of stem cell responses, from the induction of programs of differentiation (Tsai and McKay, 2000) to promote proliferation and self-renewal properties (Purpura et al., 2004). In particular, hESCs are standard cultivated as flat colonies in static adherent conditions. Typically, these colonies are maintained at an appropriate size to assure controlled self-renewal. It is well established that individual cells or small clumps do not grow efficiently while large colonies exhibit substantial levels of spontaneous differentiation (Azarin and Palecek, 2010; Bauwens et al., 2008).

In addition, the spatial distribution of the ECM within the stem cell niche in combination with these cell-cell interactions physically affects stem cell behaviour. Using soft lithography techniques, researches have investigated the influence of spatially patterned adhesion molecules on cell differentiation (McBeath et al., 2004). These patterning tools have been used to investigate cell spreading and shape on mesenchymal stem cell (MSC) differentiation, through control of the cellular cytoskeleton. MSCs patterned on larger islands of adhesion ligands, which allowed for cell spreading tended to differentiate into osteoblasts, whereas cells on smaller islands, where cells stayed rounded, differentiated into adipocytes (McBeath et al., 2004). Therefore, specific culture parameters such as the cell inoculum concentration, co-cultivation with other cell types and surface patterning/topography require optimization so that bioprocess performance can be tightly controlled, improving the robustness and reproducibility of the cultures (Figure 1.3, page 12).

3.2.4. Physical forces

A number of *in vivo* and *in vitro* studies have demonstrated that physical forces (e.g. hydrodynamic/hydrostatic, mechanical and electrical) play a key role in the development of tissues and organs during embryogenesis as well as their remodelling and growth in postnatal life. Moreover, it has been found that stem cells are sensitive to fluid flow-induced shear stress (Glossop and Cartmell, 2009), compressive and tensional strains (Haudenschild et al., 2009), cyclical stretching (Shimizu et al., 2008) and hydrostatic pressures (Liu et al., 2009). In particular, Sargent *et al* demonstrated that manipulation of hydrodynamic environments modulates the kinetic profile of gene expression and relative percentages of ESC differentiation (Sargent et al., 2010). In another study, Veraitch *et al* reported that excessive centrifugal forces up to 1000 g cause shifts in phenotype and proliferation during expansion and differentiation of ESCs (Veraitch et al., 2008).

However, relatively few information is known about the impact of these physical forces on physiological mechanisms. Recently, Stolberg *et al* proposed potential mechanisms for shear stress signalling that may play a role in endothelial differentiation trough the VEGF signalling pathway (Stolberg and McCloskey, 2009). Since scalable culture systems often employ perfusion or mixing that can apply mechanical forces to the cells, this kind of information will be extremely important to design efficient bioreactor-based strategies. The effect of shear-protection additives on hESC proliferation, viability and pluripotency will be important information for the establishment of scalable bioprocesses.

3.2.5. Physiochemical environment

The propagation and differentiation of stem cell cultures are highly dependent on the physiochemical conditions. The concentration of nutrients

and metabolites affects cell growth, viability and differentiation. In order to mimic the *in vivo* physiological environment and further improve culture performance, different operation modes can be adopted, including fed-batch and perfusion. The fed-batch strategy is often considered the most suitable for tuning cell metabolism; by providing nutrients in a rational manner, their uptake and consumption are energetically more efficient leading to reduced accumulation of metabolites in culture supernatant (Xie and Wang, 1994). However, as described above, growth factors play a crucial role in regulation of stem cell behavior. Thus perfusion mode has been preferentially adopted in the majority of stem cell bioprocesses aimed to control culture outcome, since it assures the continuous renewal of nutrients and other factors as well as the continuous removal of metabolic byproducts (Bauwens et al., 2005). Within this context, more knowledge regarding the *in vivo* stem cells microenvironment is needed, i.e. the concentration gradients existed in stem cell niches in order to understand their impact on stem cells' fate decisions.

Although typically cultivated inside of incubators operated at standard conditions of temperature (37ºC), dissolved oxygen tension (20%) and pH (7.4), stem cell expansion and differentiation potential can be enhanced at different conditions. Up to now few studies have been conducted on the effect of temperature and pH in stem cell culture. For instance, it has been shown that mesenchymal stem cell differentiation is enhanced at lower temperatures (32ºC) than in 37ºC conditions (Stolzing and Scutt, 2006) while high temperatures (39ºC) demonstrated to enhance megakaryopoiesis in CD34- enriched cord blood cells (Proulx et al., 2004). Concerning pH, it was demonstrated that high values (pH 7.60) enhance differentiation and maturation of megakaryocyte progenitors (McAdams et al., 1998) whereas low pH values (7.1) increase their expansion capacity (Yang et al., 2002). Recently, Veraitch *et al.* reported that extended

exposure of ESC cultures for 1-3 h to ambient conditions during passaging procedures (which resulted in a rapid drop in temperature and rise in pH) inhibits cell proliferation and reduces the expression levels of Oct-4 (Veraitch et al., 2008).

Stem cell niches are often located in regions of low oxygen tension (pO_2) and low pO_2 typically decreases the rate of stem cell differentiation and enhances stem cell proliferative potential (King and Miller, 2007; Millman et al., 2009). Regarding hESC culture, there is emerging evidence that reducing oxygen concentration towards physiological levels, i.e. low levels of oxygen (hypoxia – 1.5-8%) is beneficial for *in vitro* maintenance of their pluripotent status: stem cells self-renewal is supported, spontaneous (uncontrolled) differentiation is reduced and karyotypic integrity is maintained (Ezashi et al., 2005; Prasad et al., 2009), contrasting to normoxia conditions (20% oxygen). The hypothesis is that these hypoxic environments protect the ESCs from oxygen toxicity while inducing the up-regulation of an array of genes orchestrating the earliest steps of embryonic development. Nonetheless, further investigation is required to support such assumption.

Based on these findings and in an attempt to unlock the full potential of stem cells, bioprocess engineers are focused on recreating *in vitro* the dynamic environments experienced by cells *in vivo*. However, the design of such complex microenvironments is not trivial. The degree of complexity involving the incorporation of various ECM proteins, soluble factors and cell populations into different physical *stimuli* and physiochemical conditions, within a heterogeneous spatial and temporal pattern, generates a large space of solutions from which the development of new cell-based products should be initiated. From a bioprocess perspective, these microenvironments can be engineered by combining 3-D culturing approaches with bioreactor technology.

3.3. Moving stem cells from 2-D monolayers to 3-D culturing approaches

Stem cells are traditionally cultured in 2-D systems (e.g. Petri dishes, culture flasks and well plates). In particular, hESCs are propagated as colonies on top of a feeder layer of inactivated fibroblasts (Figure 1.4).

Over the last years, constant inadequacy of conventional 2-D culture systems in resembling the *in vivo* developmental microenvironment has been observed in both basic biology and tissue engineering studies. In fact, tissue-specific architecture, mechanical and biochemical cues, cell-cell and cell-matrix communications are lost under such simplified and highly biased conditions. In addition, the inherent uncontrollability, heterogeneity and low production yields associated with these systems have made them unattractive and unsuitable for clinical and industrial applications.

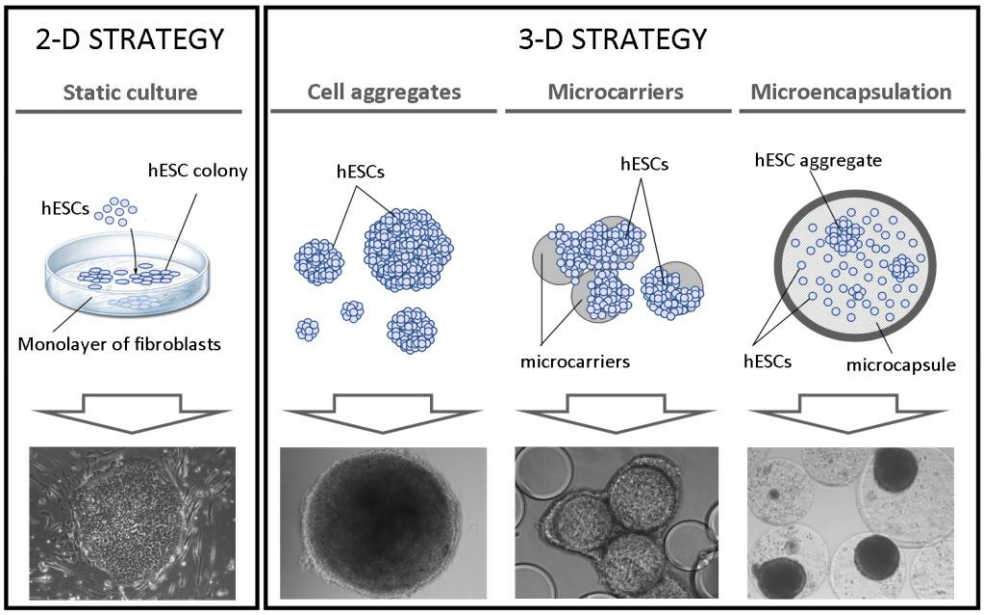

Figure 1.4. Two-dimensional (2-D) and three-dimensional (3-D) strategies for cultivation of human embryonic stem cells (hESCs).

Moving stem cells from 2-D cell monolayers to 3-D culturing strategies is imperative to enhance cells' performance and fully exploit cells' potential. The general recognition that spatial arrangement and directional cues have an important role in stem cells behaviour contributed for the acceptance of 3-D cultures as the most suitable system to mimic stem cells' native microenvironment. By providing a cellular context closer to what actually occurs in native microenvironment, these strategies can significantly improve cell's viability, identity and function (Cukierman et al., 2002; Lund et al., 2009; Pampaloni et al., 2007). In summary, engineered 3-D microstructures have the potential to provide a higher degree of efficiency, robustness, consistency and predictability to the cultures.

This section will address current 3-D culture strategies that could be used to generate large numbers of pluripotent hESCs and/or their derivatives with potential application in regenerative medicine and drug discovery. It is important to highlight that, an optimal hESC based bioprocess capable of embracing all the applications of these cells does not exist so far. Nonetheless, the knowledge gained during the last decades with murine ESCs (mESCs) and other stem cell model systems (e.g. human teratocarcinoma stem cells), in which the quantitative characterization of expansion and differentiation processes is included, have been providing important insights for the development of robust hESC production platforms.

A variety of 3-D microstructures are currently established for stem cell expansion and/or differentiation. Self-aggregated spheroids (3-D cell aggregates), cell immobilization on microcarrier and cell encapsulation in biomaterials, are some examples. The main benefits and disadvantages of 2-D and 3-D strategies for stem cell cultivation are listed in Table 1.3.

Table 1.3. List of advantages and disadvantages of different culture systems for stem cell bioprocessing.

Culture Strategy	Advantages	Disadvantages
2-D Culture Static Cultures	• easy visualization and cell morphology monitorization • easy handling • affordable system • ideal for small scale studies	• low reproducibility • low scalability •difficult to control specific culture parameters and diffusion gradients • low cell production yields • limitation in resembling in vitro tissues
Cell aggregates	• easy handling • scalable system • high reproducibility • 3- D cell-cell contact is preserved • can mimic stem cells' native microenvironment • high differentiation efficiency • high cell production yields	• difficult to control culture outcome due to the occurrence of uncontrolled/spontaneous differentiation (EB formation) • aggregate size (important to avoid diffusion gradients inside the aggregate structure that lead to necrotic centres and/or spontaneous differentiation) • single cell harvesting (difficult to dissociate aggregates without compromising cell viability) • cell damage due to physical forces (hydrodynamic shear, perfusion flow)
Microcarriers Non-porous	• easy handling • scalable system • high reproducibility • easy visualization and cell morphology monitorization • No limitations in mass and gas diffusion • high surface to volume ratio (able to support high cell densities, reduce the process cost) • high cell production yields	• microcarrier agglomeration (important to avoid diffusion gradients inside the cell-microcarrier aggregate structure that lead to necrotic centres and/or spontaneous differentiation) • cell-bead separation step required • cell damage due to physical forces (hydrodynamic shear, perfusion flow) • costs associated to material (microcarrier)
Porous	• easy handling • scalable system • high reproducibility • high surface to volume ratio (able to support high cell densities, reduce the process cost) • high cell production yields • protection from physical forces (hydrodynamic shear, perfusion flow)	• difficulty in culture visualization and cell morphology monitorization • Limitations in mass and gas diffusion inside the pores that lead to necrotic centres and/or spontaneous differentiation • cell harvesting limitation (except for biodegradable supports) • cell-bead separation step required • costs associated to material (microcarrier)
Cell Microencapsulation	• easy handling • scalable system • high reproducibility • high surface to volume ratio (able to support high cell densities, reduce the process cost) • high cell production yields • protection from physical forces (hydrodynamic shear, perfusion flow) • 3- D cell-cell and cell-matrix contacts are preserved, mimicking stem cells' native microenvironment • biomaterial can be engineered to improve cell culture performance • process can be integrated in transplantation studies	• difficulty in culture visualization and cell morphology monitorization • Limitations in mass and gas diffusion inside the pores that lead to necrotic centres and/or spontaneous differentiation • cell harvesting (decapsulation protocol could compromise cell viability) • costs associated to encapsulation equipment/process and biomaterials

3.3.1. Cell aggregates

By aggregation into spheroids, cells can re-establish mutual contacts and specific microenvironments that allow them to express a tissue-like structure. Within this context, the cultivation of stem cells as 3-D aggregates has been extremely explored during the last decades proving to be an efficient system for expansion/differentiation of progenitor cells, such as human neural precursor cells (Baghbaderani et al., 2008; Baghbaderani et al., 2010), pancreatic cells (Chawla et al., 2006) and hepatocyte progenitors (Gerlach et al., 2003; Miranda et al., 2009).

For ESCs, the 3-D aggregate culture strategy is usually associated with differentiation; the most robust method for generating differentiated cells from ESCs is through the formation of embryoid bodies (EBs), where ESC cultured in suspension self-aggregate and spontaneously differentiate into multiple tissues (Dang et al., 2004). EB differentiation has been shown to recapitulate aspects of early embryogenesis, including the formation of a complex 3-D arrangement where cell-cell and cell-matrix interactions are thought to support the development of three embryonic germ layers and their derivatives (Itskovitz-Eldor et al., 2000; Keller, 1995).

The main limitation of this system is, in fact, the inefficient control of stem cell expansion or in directing stem cell differentiation towards a specific lineage, thus resulting in a mixture of different cell types. This drawback demands the need of efficient integrative downstream approaches to further purify the culture outcome into a desired cell type population.

Cormier et al. were the firsts who developed a system for the expansion of undifferentiated mESCs as aggregates in stirred bioreactors, achieving 31-fold expansion during 5 days without compromising stem cell characteristics (Cormier et al., 2006). These results were very encouraging, contributing for the implementation of improved and scalable protocols for

expansion of pluripotent stem cells. One year later, zur Nieden demonstrated that mESCs could be maintained for a total of 28 days in these culture systems by repeated aggregate dissociation (zur Nieden et al., 2007).

Besides propagation, many studies have been performed in directing differentiation of mESCs aggregates into a specific cell lineage (revised in (Jensen et al., 2009; King and Miller, 2007; Ulloa-Montoya et al., 2005).The knowledge gained with these model systems combined with developments in fundamental cell biology contributed to the design of controlled bioprocesses for hESCs. During the last 2 years, significant efforts have been made in 3-D aggregate culture systems for controlled expansion of undifferentiated hESCs and their directed differentiation into functional cell types (summarized in Table 1.4).

3.3.2. Cell immobilized in microcarriers

A microcarrier is a support matrix that allows the growth of anchorage-dependent cells in suspension systems. Microcarrier cultures are characterized by high surface-to-volume ratio, accommodating higher cell densities than those obtained in static cultures; the area available for cell growth can be adjusted easily by changing the amount of microcarriers, which further facilitates the process scale-up. From industrial/ commercial/ clinical perspectives, this feature has a tremendous impact in reducing the costs of cell manufacturing by reducing the amount of media, growth factors and other expensive supplements required in stem cell cultivation. For each stem cell type and bioprocess it is important to optimize specific parameters including microcarrier type, concentration and inoculum density.

Table 1.4. Summary of the studies involving the cultivation of hESCs as aggregates.
(STLV: slow turning lateral vessel; DMEM-KO: knockout Dulbecco's modified Eagle's medium; KO-SR: knockout serum replacement; FBS: foetal bovine serum; EB: embryoid body; IL6RIL6: interleukin-6 receptor fused to interleukin-6.; RI: Rock inhibitor)

Culture Conditions	Results		Ref.
	Expansion	Differentiation	
System: STLV Medium: DMEM-KO, FBS and supplements Strategy: EB culture	70-fold in 28 days (max: 36x10^6 cell/mL)	EB formation –No specific cell lineage differentiation	(Gerecht-Nir et al., 2004a)
System: spinner flasks Medium: DMEM, FBS and supplements Strategy: EB culture	15-fold in 21 days (max: 2-3 x10^5 cell/mL)	hematopoietic progenitors 5-6% at day 14	(Cameron et al., 2006)
System: perfused and dialyzed STLV bioreactors Medium: *DMEM-KO ,KO-SR and supplements* Strategy: *EB culture*	-	Efficient EB formation and rapid EB differentiation into neural cells	(Come et al., 2008)
System: STLV spinner flask ball impeller spinner flask with paddle impeller Medium: *DMEM-KO ,KO-SR and supplements* Strategy: EB culture	1.2-fold in 10 days 6.4-fold in 10 days 2.2-fold in 10 days	EB formation –No specific cell lineage differentiation	(Yirme et al., 2008)
System: spinner flask with triangle impeller and glass-etched baffles Medium: mEFs conditioned medium Strategy: hESC aggregates in 10% matrigel	5.6-fold in 10 days (max: 3.4 x10^6 cell/mL)	-	(Kehoe et al., 2009)
System: spinner flasks uncontrolled conditions stirred bioreactor 21% oxygen stirred bioreactor 4% oxygen Medium: DMEM-KO, FBS and supplements Strategy: patterned hESC colonies-EBs	Max cell concentration: 2.2 x10^5 cell/mL day 16 4.0 x10^5 cell/mL day 16 5.2 x10^5 cell/mL day 16	Cardiomyocyte (day 16) 23.7% 48.3% 48.8%	(Niebruegge et al., 2009)
System: spinner flasks System: mTeSR, 0,1nM Rapamycin ,10 µM Rocki Strategy: Treatment with RI after single cell dissociation	25-fold in 6 days (max: 4.5 x10^5 cell/mL)	-	(Krawetz et al., 2009)
System: spinner flasks with a bulb-shaped pendulum Medium: mTeSR medium with 10µM Rocki Strategy:*Heat shock and RI treatment after single cell dissociation*	2-fold in 7 days (>2x10^6 cells/mL)	-	(Singh et al., 2010)
System:Erlenmeyer flask Medium: *mTeSR, 10 µM ROCKi* Strategy: Treatment with Rocki after single cell dissociation	21.6-fold in 4 days (max: 7-8 x10^5 cell/mL)	EB formation	(Olmer et al., 2010)
System:Erlenmayer flasks Medium: *DMEM-KO , 100 pg/ml IL6RIL6 chimera* Strategy: *inoculation of hESC clumps*	25-folds in 10-11 days (max: 9 x10^5 cell/mL)	-	(Amit et al., 2010)

A wide range of microcarrier types are commercially available today; supports can be porous or non-porous, composed by gelatin, glass, collagen, cellulose, presenting dimensions within the range of 10 to 6000 µm. In addition, these microcarriers can be functionalized with different coating materials (ECM proteins, small molecules) in order to further improve cell culture performance (attachment and growth). Thus, microcarrier technology allows the flexibility of culturing the cells in different conformations and on different matrices.

Cells cultured in macroporous beads (e.g. Cytopore2, CultisphereS) are cultured in a 3-D system, protected from the shear stress, although the diffusion of oxygen and nutrients within the bead could be limited. These systems have been used for the expansion and differentiation of mouse embryonic stem cells (Akasha et al., 2008; Fernandes et al., 2007; Storm et al., 2010) and for propagation of MSCs (Eibes et al., 2010).

In non-porous microcarriers (e.g. Cytodex 1 and Cytodex 3, Hillex II), cells are attached to the surface of the beads, assuming a similar configuration to that of 2-D monolayers. Adult stem cells, such as mesenchymal stem cells, demonstrated higher expansion yields while keeping their phenotype and differentiation potential on non-porous microcarriers (Sart et al., 2009). One of the challenges that still need to be addressed is the optimization of cell harvesting protocols after expansion/differentiation process, to guarantee efficient cell-bead separation and high cell recovery yields without compromising their viability, potential and/or functionality.

Recent results have shown that hESCs exhibit improved cell growth and retain their differentiation potential when cultured on dextran or cellulose-based microcarrier supports, coated with matrigel or denatured collagen (Lock and Tzanakakis, 2009; Nie et al., 2009; Oh et al., 2009; Phillips et al., 2008). Seeding hESC as single cells into microcarriers avoided formation of EBs and the consequent uncontrolled differentiation. Noteworthy is the

generation of 3-D hESC-microcarriers aggregates in culture upon microcarrier colonization (Figure 1.4). This 3-D cell growth results in additional increase in cell yields, when compared to 2-D culture systems. In this particular case, the control of microcarrier clumping will be critical to avoid the formation of larger aggregates that could lead to diffusion limitations. A summary of the studies performed using microcarrier technology for the production of hESC-based products as well as the main results obtained are indicated in Table 1.5.

Table 1.5. Summary of the studies involving the cultivation of hESCs immobilized in microcarriers. (mEFs: mouse embryonic fibroblasts; DMEM-KO: knock out Dulbecco's modified Eagle's medium; KO-SR: knock out serum replacement;)

Culture Conditions	Results		Ref.
	Expansion	Differentiation	
Microcarriers: trimethylamonium-coated polydtyrene microcarriers (Hillex II) Medium: mEFs conditioned medium System: ultralow attachment plates	2.5-fold in 5 days ($0.2x10^6$ cell/mL)	-	(Phillips et al., 2008)
Microcarriers: Cytodex^{TM}3 microcarriers coated with Matrigel Medium: mEFs conditioned medium System: ultra low well plates	3.4-fold in 2.5 days		(Nie et al., 2009)
Microcarriers: Hyclone microcarriers coated with matrigel Medium: mEFs conditioned medium/Differentiation medium System: 50 mL spinner flasks	34- to 45- fold in 8 days ($1x10^6$ cell/mL)	Differentiation to definitive endoderm >80% efficiency	(Lock and Tzanakakis, 2009)
Microcarriers: Cytodex^{TM}3 microcarriers Medium: mEFs conditioned medium System: spinner flasks	6.8-fold in 14 days ($1.5\ x10^6$ cell/mL)	-	(Fernandes et al., 2009)
Microcarriers: Matrigel-coated cellulose microgranular cylindrical Medium: mEFs conditioned medium System: spinner flasks	5.8-fold in 5 days ($3.5x10^6$ cell/mL)	-	(Oh et al., 2009)
Microcarriers: Cultisphere S Medium: DMEM-KO, KO-SR and supplements System: spinner flasks	10- fold in 7 days ($3.5x10^6$ cell/mL)		(Storm et al., 2010)
Microcarriers: TSKgel Tresyl-5PW (TOSOH-10) coated with laminin Medium: Differentiation medium System: spinner flasks	-	20% cardiomyocytes day 16 3-fold expansion in 16 days ($2.14x10^5$ cardiomyocyte/mL)	(Chen et al., 2010)

To overcome certain problems encountered in cell therapy, particularly cell survival, lack of cell differentiation and integration in the host tissue, the use of pharmacologically active microcarriers (PAM) has been explored (revised in Delcroix et al., 2010; Hernandez et al., 2010). These biodegradable particles made with poly(D,L-lactic-*co*-glycolic acid) (PLGA) and coated with specific adhesion molecules serve as a support for survival and differentiation of the transported cells as well as their microenvironment, ultimately enhancing graft integration.

3.3.3. Encapsulated cells

The main benefit of cell encapsulation technology is the possibility of designing the scaffold environment with specific biomaterials that exhibit a wide range of mechanical/chemical properties, correlating to the properties of native tissues. Such tailored microenvironments may be more suitable for the self-renewal of stem cells, for directing their differentiation into specialized cell types, for promoting the organization of cells in 3-D configurations similar to those established *in vivo*.

Within this context, several scaffolds/biomaterials have been used to enhance the culture of hESCs including alginate (Siti-Ismail et al., 2008), poly (lactic-co-glycolic acid)/poly(l-lactic acid) scaffolds (Levenberg et al., 2003) and hydrogels of agarose (Dang et al., 2004), chitosan (Li et al., 2010), synthetic semi-interpenetrating polymers (Li et al., 2006), hyaluronic acid (Gerecht et al., 2007a) and a natural components from the ECM (Yang et al., 2010) (also described above, Table 1.2).

Many different kinds of encapsulation systems have been studied. The entrapment of cells in microcapsules has demonstrated several advantages in stem cell bioprocessing since the small size and spherical capsules offer an optimal surface to volume ratio and appropriate diffusion capacity of nutrients, growth factors and gases. Cell microencapsulation technology is

also a valuable tool for improving cell yields since it protect cells from the harmful effects associated to shear stress and avoid excessive agglomeration of cell aggregates. Therefore, this approach has been adopted and combined with bioreactor systems (stirred vessels, HARV, perfusion bioreactors) to enhance the formation of tissues and the differentiation of stem/progenitor cells to myocardium (Bauwens et al., 2005), hepatocytes (Maguire et al., 2007; Maguire et al., 2006), pancreatic islets (Lee et al., 2009; Wang et al., 2009), bone (Goldstein et al., 2001), cartilage (Kuo et al., 2006), hematopoietic cells (Liu and Roy, 2005), neuronal cells (Delcroix et al., 2010) and vascular grafts (Nieponice et al., 2008). Up to now, there are just three studies that explored the use o microencapsulated technology in hESC cultivation (Table 1.6).

Table 1.6. Summary of the studies involving the cultivation of microencapsulated hESCs. (PLL: poly-L-lysine ; EB: embryoid body)

Culture Conditions	Results		Ref.
	Expansion	Differentiation	
Microcapsules: 1,1% calcium alginate; (diameter: 1mm) System: static culture	Efficient proliferation hESCs maintained their undifferentiated state up to 260 days	-	(Siti-Ismail et al., 2008)
Microcapsules: 1,1% calcium alginate and 0,1 % gelatin; (diameter: 400-600 µm) System: static culture	70-80% cell viability Cluster formation Cell growth in 8 days	Directed differentiation to definitive endoderm cells	(Chayosumrit et al., 2010)
Microcapsules: 1.5% calcium alginate coated with PLL; liquid core capsules (diameter: 500-600 µm) System: static culture and spinner vessels	9-fold in 15 days ($16x10^4$ cells/mL) >85% viability	Differentiation into cardiomyocytes via EB culture	(Jing et al., 2010)

It is important to highlight that microencapsulation technology will also contribute for the success of transplantation experiments. In contrast to cells in suspension, encapsulated tissue constructs are less susceptible to immune rejection, their delivery is better target and the *in vivo* degradation kinetics can be tuned permitting a more efficient and functional integration of cells in the host organ (Delcroix et al., 2010; Murua et al., 2008).

3.4. Bioreactors for stem cell cultivation

Bioreactors have been, and still are, extensively used in chemical/biological industries for the production of enzymes, antibodies, viruses, recombinant proteins amongst many other products. The knowledge accumulated from the years facilitated their transition to stem cell bioengineering, in which the cells are the main products. At present, there is a large range of designs available, which ranges from fluidized and packed bed bioreactors to airlift, hollow fibre and disposable wave bioreactors (Table 1.7). In the particular field of stem cell research, microfluidic devices, rotary cell culture (RCC) systems and stirred culture vessels have been the main bioreactors explored till the moment (Figure 1.5).

Bioreactors for stem cells are designed to accurately control/regulate the cellular microenvironment that supports cell viability and provides spatial and temporal control of signalling. These fully controlled bioreactors should allow a rapid and controlled cell expansion and/or differentiation, an efficient local exchange of gases (e.g. oxygen), nutrients, metabolites and growth factors as well as the provision of physiological *stimuli*.

In order to successfully translate stem cell technologies from bench to bedside, the clinical efficacy of a stem cell-based product needs to be accompanied by a scalable and cost effective manufacturing process. In addition, it must comply to the evolving regulatory framework in terms of quality control and good manufacturing practices (GMP) requirements. In the end, by generating and maintaining a controlled culture environment, stem cells bioreactors represent a key element for the development of automated, standardized, traceable, cost-effective, and safe manufacturing processes for stem cell-based products.

Table 1.7. Culture systems for stem cell expansion and differentiation (Adapted from Placzek et al., 2009; Azarin and Palecek, 2009). (Abbreviations: Monit- Monitoring, 2D- two dimensional, 3D- three dimensional; perf-perfusion).

Bioreactor type	Culture surface area ($x10^3$ cm^2/L)	Production ($x10^5$ cell/mL)	Easy of sampling	Non-invasive sampling	Monit. & Control	Mass transfer	Easy to scale-up	Shear stress (dyn/cm^2)	Operation mode		Culture approach		Commercially available	
									batch	perf	2D	3D	Company	Price ($/mL)
Static systems (T-flasks, dishes)	0.29	0.1	high	no	low	low	low	0	yes	no	yes	no	Corning	0.15
Stirred culture systems	2.8	1-10	high	yes	high	high	high	2-40	yes	yes	yes	yes	Kimble/Kontes	0.3
Perfusion chamber	18	10-100	medium	no	medium	medium	medium	1-5	no	yes	no	yes	Aastrom Biosciences Inc.	43
Perfusion hollow fibre	100-200	100-200	low	no	low	medium	medium	0	no	yes	yes	yes	FiberCell Systems, Inc.	3.3
Rotating wall vessel	18-22	n.a.	medium	no	medium	medium	low	0.5-2	no	yes	yes	yes	Synthecon, Inc	25
grooved bioreactor	18-20	10-100	medium	no	medium	medium	medium	0.1-0.5	no	yes	no	yes	Baxter International	40-45
Packed bed (Bellocel)	18	1.5-200	medium	no	low	medium	high	1-5	no	yes	yes	yes	Cesco Bioengineering Co., Ltd	3.0
Airlift	2800	0.5	medium	yes	high	high	medium	10-30	yes	yes	yes	yes	Kimble/Kontes	0.9
Disposable bag bioreactors (wave bioreactor)	n.a.	10-20	high	yes	high	medium	high	0.1-0.5	yes	yes	yes	yes	GE Healthcare	0.25

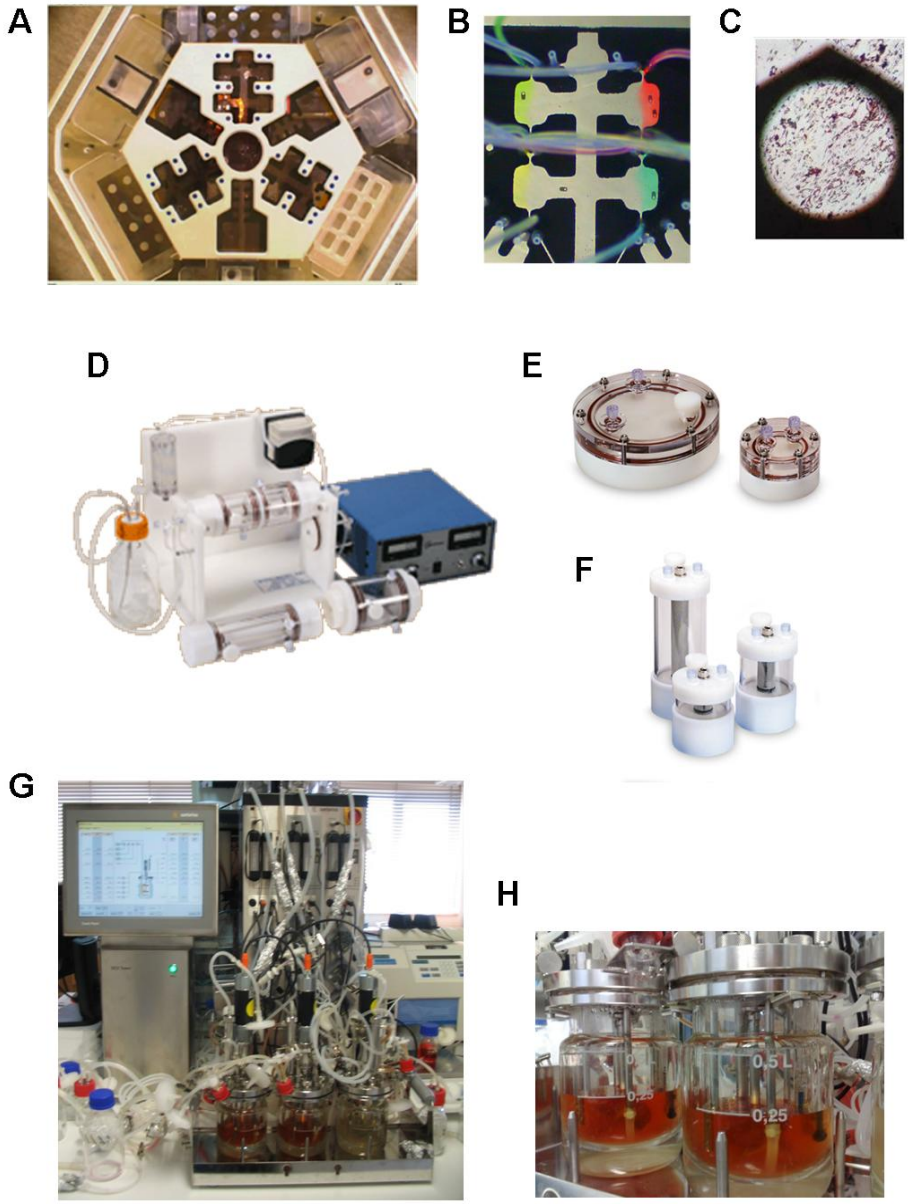

Figure 1.5. Bioreactors used for stem cell cultivation. (A-C) Micro-bioreactor developed within the scope of CellPROM European project (www.cellprom.net): (A) MagnaLab central unit, (B) chip compartment, (C) cell carrier; (D-F) Rotary cell culture system (www.synthecon.com): (D) bioreactor unit, (E) high aspect rotating vessels, (F) slow turning lateral vessels. (G-H) Fully controlled stirred tank bioreactor (BIOSTAT® Qplus): (G) bioreactor unit, (H) stirred vessels.

3.4.1. Microfluidic culture systems

Microfluidic devices, or micro-bioreactors, are efficient small-scale systems mainly used for the optimization of culture conditions for cell expansion and differentiation while also providing the precise control over the cell microenvironment (Azarin and Palecek, 2010; Placzek et al., 2009) (Figure 1.5A-C). Arrays of micro-bioreactors have been developed to study growth and differentiation of hESC and ASC in a 3-D perfusion system (Cimetta et al., 2009; Fong et al., 2005; Gottwald et al., 2008; Zhao et al., 2009). The microenvironment can be controlled by adjusting specific operating parameters such as the perfusion rate, resulting in a high-throughput system for evaluating the effects of concentration gradients of soluble factors on various cell processes. However, the main limitations of these culture systems are low scalability and the high shear stress associated to perfusion as well as and the continuous removal of important factors secreted by the cells that could ultimately compromise stem cell performance.

3.4.2 Rotary cell culture systems

Developed by NASA, RCC bioreactors (which includes STLV- slow turning lateral vessel and HARV- high aspect rotating vessel) are composed by a rotating 3-D chamber in which cells remain suspended in near free-fall, simulating microgravity conditions (Figure 1.5D-F). These low shear stress bioreactors can provide a well mixed environment for cell growth as well as efficient gas transfer through a silicon membrane. Rotary cell culture systems have been used for expansion of cells as of human EBs, and for multiple ASC using scaffolds (Come et al., 2008; Gerecht-Nir et al., 2004a; King and Miller, 2007).

Amongst the main disadvantages of RCC are the limited control of aggregate size and nutrient/gas concentrations throughout the vessel. This may result in the formation of necrotic centers, leading to cell death inside the aggregates, and uncontrolled microenvironments, caused by the concentration gradients resulted from mass transfer limitations. In addition the working volume of these bioreactors is still low, thus limiting their use in a clinical and/or larger scale.

3.4.3. Stirred culture vessels

Stirred culture vessels, including spinner vessels and stirred tank bioreactors (Figure 1.5G-H), are scalable and hydrodynamically well characterized systems with simple design and operation. The main characteristic of these bioreactors is the possibility of culturing cells in a dynamic stirred environment, overcoming the mass transport and gas transfer limitations of static and other bioreactor systems (Table 1.7). Here, the impeller design and ranges of stirring rate should be delineated carefully for each case study since each stem cell type has different sensitivities/necessities in terms of the shear stress. Another important feature of these bioreactors is the feasibility to perform non-invasive sampling thus enabling the continuous monitorization/characterization of the stem cell culture status/performance which is critical for process optimization (Figure 1.6).

In particular, fully controlled stirred tank bioreactors provide an automated control of the environment, allowing the on-line monitoring and control of specific culture variables (temperature, pH, dissolved oxygen, nutrients) that can affect stem cell self-renewal and directed differentiation, ultimately improving culture outcome and ensuring reproducibility (Figure 1.6).

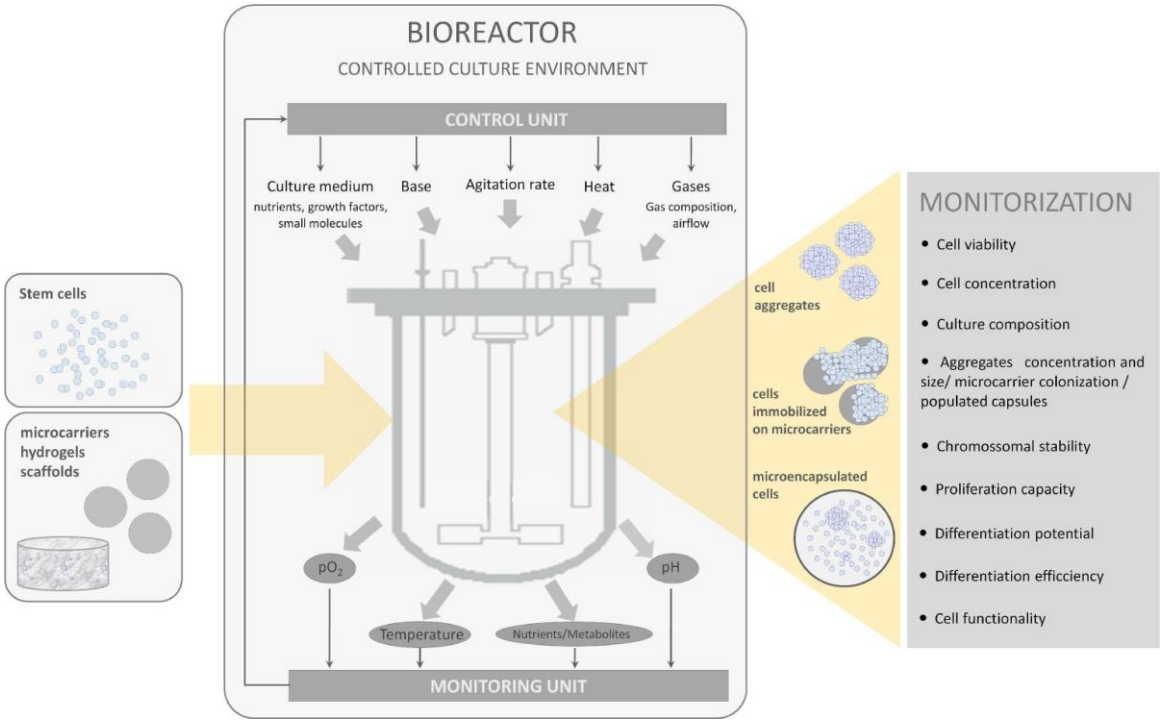

Figure 1.6. Schematic representation of stirred tank bioreactor system for stem cell cultivation. Fully controlled stirred tank bioreactors provide an automated control of the environment (temperature, pH, pO_2 as well as nutrients/metabolites) mandatory for reproducible stem cell cultivation. Stirred culture vessels are scalable and hydrodynamically well characterized and enable easy non-invasive sampling for continuous culture monitorization (e.g. cell concentration/viability, culture characterization, differentiation potential, cell function). These bioreactors provide the operator with the flexibility of various modes including the culture of cells as aggregates, on microcarriers or in microcapsules. Adapted from *(Wendt et al., 2009)*

These bioreactors are highly flexible as they can operate in different culture operation modes (batch, perfusion), can be adapted to different type of bioprocesses (stem cell expansion and/or differentiation) and can be accommodated to different 3-D culture strategies (cell aggregates, microcarriers, microencapsulated cells), presenting widespread potential in stem cell bioengineering (Jang et al., 2010; Niebruegge et al., 2009).

The key studies reporting the use of stirred culture bioreactors in hESC expansion and differentiation are listed above in Tables 1.4, 1.5 and 1.6. One of the main limitations of stirred culture vessels is the hydrodynamic stress promoted by stirring, as described above. In addition, the minimal volume required to set up the experiments is very high (approximately 50 mL), demanding higher starting cell numbers, increasing the costs associated to optimization studies and compromising the use of stirred bioreactors for high-throughput applications.

4. SCOPE OF THE THESIS

This thesis focused on the development of robust and scalable systems for the efficient production of cell-based products, capable of generating relevant numbers of well characterized cells for therapeutical and/or pharmacological applications. More specifically, the cultivation of stem cells in a 3-D culturing approach using stirred tank bioreactors was explored. The overall goal was to obtain robust protocols for the expansion of hESCs which can ensure the production of pluripotent stem cells in high quality and relevant quantities.

To achieve this, an integrated approach was developed by evaluating different 3-D culturing strategies (cell aggregates and cells immobilized to microcarriers) and addressing specific bioprocessing parameters namely inoculum concentration, microcarrier type and culture operation mode. On a

first step, two model systems were used to establish preliminary strategies for the production of (i) undifferentiated stem cells as well as (ii) neuron derived-stem cells, namely

(i) rat pancreatic stem cells (rPSCs), due to their great potential for self-renewal and multilineage differentiation. In addition, rPSCs showed spontaneous differentiation into lineages of the three germ layers (Kruse et al., 2004). This plasticity potential makes them an appealing source for cell replacement therapies and tissue engineering applications;

(ii) human embryonal carcinoma stem cell line NTera-2/cl.D1 (NT2), since it presents similar characteristics with undifferentiated hESCs including the expression of stem cell markers, high self-renewal ability and pluripotency. Moreover, NT2 cells are also a valuable model for human neuronal differentiation *in vitro*, showing patterns of morphological differentiation similar to the ones present *in vivo* neurogenesis. The neurons derived from this cell line have been successfully used in transplantation and toxicology studies (Kondziolka and Wechsler, 2008), providing a promising material for cell therapy and drug screening investigations.

The knowledge gained from these systems contributed for understanding better the complexity of hESC culture and fulfill the final aim of this thesis which was the implementation of robust bioprocesses for the production of hESCs with high cell viabilities and expansion yields and without compromising their undifferentiated phenotype and pluripotency.

Taking into account that oxygen and medium perfusion have shown to be essential parameters in hESC culture (Placzek et al., 2009), the possibility of cultivating hESCs in environmentally controlled stirred tank bioreactors, where process automation and tight monitorization/control of the culture

environment are strictly ensured, was evaluated aiming to improve the expansion yields of pluripotent hESCs.

One of the drawbacks associated to the combination of these 3-D approaches with stirred tank bioreactors in hESC cultivation is the hydrodynamic stress caused by stirring. Therefore the effect of alginate microencapsulation technology was investigated not only to protect cells from the shear stress and to avoid the commonly observed aggregate clumping or microcarrier agglomeration but also as a main strategy to facilitate bioprocess integration with cryopreservation protocols.

A schematic representation of the main aims proposed for this thesis as well as the strategies that will be employed to address them are summarized on Figure 1.7.

The development of robust strategies for the scalable production of complex stem cell systems addressed in this thesis will facilitate the transition of stem cell-based products for a broad spectrum of applications in regenerative medicine, tissue engineering and *in vitro* toxicology.

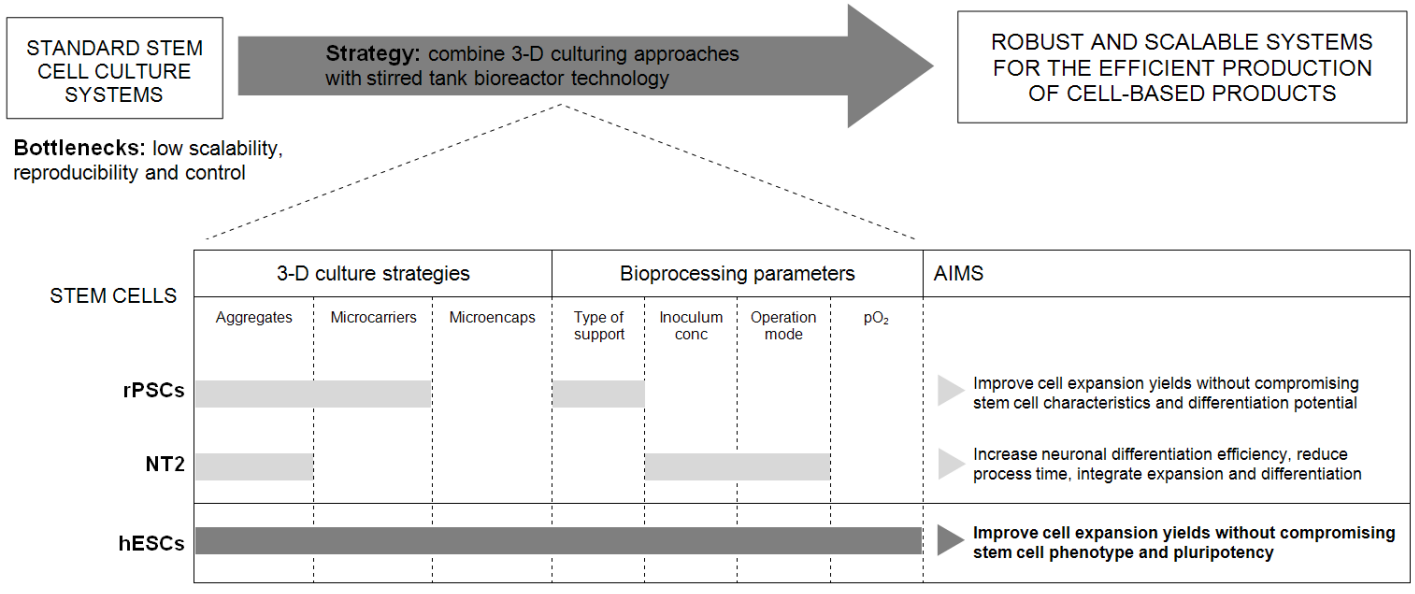

Figure 1.7. Diagram of the main aims proposed for this thesis. Highlighted in grey are the strategies addressed for each stem cell system. (Abbreviations: hESCs-human embryonic stem cells; Microencaps-microencapsulation; NT2-human embryonal carcinoma stem cells, rPSCs- rat pancreatic stem cells).

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CHAPTER 2

EXPANSION OF ADULT PANCREATIC STEM CELLS IN STIRRED TANK BIOREACTORS

This chapter was based on the following manuscript:

Serra, M., Brito, C., Leite, S.B., Gorjup, E., von Briesen, H., Carrondo, M.J. and Alves, P.M., 2009. Stirred bioreactors for the expansion of adult pancreatic stem cells. Ann. Anat. 191, 104-115

ABSTRACT

Adult pluripotent stem cells are a cellular resource providing unprecedented potential for cell therapy and tissue engineering. Complementary to this promise, there is a need for efficient bioprocesses for their large expansion and/or differentiation.

Within this goal, our work was focused on the development of 3-D culture systems for the controlled expansion of rat pancreatic stem cells (rPSCs). For this purpose, two different culturing strategies were evaluated, using spinner vessels: cell aggregated cultures *versus* microcarrier technology. The use of microcarrier supports (Cytodex 1 and Cytodex 3) rendered expanded cell populations that retained their self-renewal ability, cell marker, and the potential to differentiate into adipocytes. This strategy overcame the drawbacks faced by aggregates in culture, revealed unfeasible as cells clumped together, did not proliferate and lost rPSC marker expression. Furthermore, the results obtained showed that, although both microcarriers tested herein were suitable to sustain cell expansion, Cytodex 3 provided a better substrate to promote cell adherence and growth.

For the last approach, the potential of bioreactor technology was combined with the efficient Cytodex 3 strategy; under controlled environment, cell growth was more efficient, as shown by faster doubling time, higher growth rate and higher fold increase in cell concentration, when compared to spinner cultures. This study describes a robust bioprocess for the controlled expansion of adult rPSC, representing an efficient starting point for the development of novel technologies for cell therapy.

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1. INTRODUCTION

The potential of adult stem cells (ASCs) to differentiate was thought to be restricted to cell types related to the tissues in which they reside, as their primary role is in tissue homeostasis and regeneration (Gokhale and Andrews, 2006). However, this concept has been challenged by the isolation of new lineage classes of uncommitted pluripotent stem cells, with a remarkable versatility in their differentiation potential, including pancreatic stem cells (PSCs) (Kruse et al., 2004, 2006). PSCs were able to growth for more than 140 passages maintaining the expression of typical stem cell markers (alkaline phosphatase, SSEA-1, Oct-4 and Nestin), as demonstrated by immunocytochemistry and RT-PCR (Kruse et al., 2006). PSCs were shown to possess great potential for self-renewal and multilineage differentiation. These cells showed spontaneous differentiation into lineages of the three germ layers; when cultured in hanging drops, PSCs formed organoid bodies, three-dimensional aggregates that contained cells of different lineages (Kruse et al., 2004, 2006). This PSC plasticity makes them an appealing source for cell replacement therapies and tissue engineering.

Complementary to the promising potential of pluripotent stem cells, there is a need for efficient culture systems for their large expansion and/or differentiation. The past years have witnessed an increased number of studies geared towards this goal. In particular, stirred suspension bioreactors have gained special interest due to their advantageous characteristics: they are hydrodynamically well characterized, easy to scale-up, enable culture homogeneity and continuous monitoring and control of the culture parameters (Ulloa-Montoya et al., 2005; King and Miller, 2007). So far, these systems have been used for a wide range of applications: (i) to culture embryoid bodies derived from mouse and human embryonic stem cells (ESCs) and differentiate them into hematopoietic and

cardiac lineages (Dang et al., 2002; Cameron et al., 2006); (ii) to expand undifferentiated murine ES cells as aggregates (Cormier et al., 2006, zur-Nieden et al., 2007); (iii) to enhance expansion and neuronal differentiation of human teratocarcinoma stem cells (Serra et al., 2007); (iv) to culture several types of tissue-specific ASCs as 3-D aggregates (Gilbertson et al., 2006; Youn et al., 2006; Chawla et al., 2006). Several research groups have also employed stirred bioreactors to culture ESCs using microcarriers as substrate to support attachment and growth (Abranches et al., 2007; Fok and Zandstra, 2005). However, many challenges remain as no studies focused on large expansion of pluripotent ASCs have been reported so far.

Herein, the feasibility of scaling-up adult PSCs expansion was assessed by testing two different approaches: cell aggregated cultures *versus* microcarrier technology.

The present work describes, for the first time, an efficient bioprocess for the controlled expansion of rat PSCs using stirred bioreactors, overcoming one of the drawbacks of stem cell technology.

2. MATERIAL AND METHODS

2.1. Cell line

The rPSC cell line (RSAPank) was derived from rat exocrine pancreas: acini from male Sprague Dawley rats were isolated at Fraunhofer Institute of Biomedical Engineering-University of Lübeck and purified as described previously (Kruse et al, 2004, 2006).

2.2. Cell culture in static adherent conditions

Rat PSCs were routinely cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% of foetal bovine serum (FBS-

Gold, PAA) and 100 U/mL penicillin-streptomycin (P/S, Invitrogen), at 37ºC, in a humidified atmosphere of 5% CO_2. Cells from passage 21 to passage 24 were used for all stirred culture experiments.

2.3. Cell culture in stirred conditions

Suspension studies were performed in spinner vessels (Wheaton, USA) of 125 mL volume (working volume - 100mL) incubated at 37ºC in a humidified atmosphere of 5% CO_2. Two different cultivation strategies were carried out - cells cultured as aggregates (Strategy 1) and immobilized in microcarrier supports (Strategy 2).

Strategy 1- Aggregates: Spinner vessels equipped with ball or paddle impellers were tested. rPSCs were inoculated at a concentration of $4x10^5$cell/mL, in 70 mL of DMEM supplemented with 15% of FBS-Gold and 100 U/mL P/S, and stirred at 50 rpm. After 6 h, culture medium was added to yield a final volume of 100 mL and the percentage of serum adjusted to 10%. During cultivation time, the agitation rate was changed from 60 to 120 rpm to avoid cell damage or aggregate clumping.

Strategy 2- Microcarriers: Two types of microcarriers were tested, Cytodex 1 and Cytodex 3 (GE Healthcare), both at a concentration of 3 g/L (dry weight). The microcarriers were prepared and sterilized according to the manufacturer's recommendations. Cell inocula ($1x10^5$cell/mL) were obtained from adherent rPSCs routinely cultured in static adherent conditions, harvested by trypsinization, collected by centrifugation, resuspended in 5 mL culture medium (DMEM supplemented with 10% FBS-Gold and 100 U/mL P/S), and immediately transferred to spinner vessels. Immobilization in the microcarriers was carried out for 4-5 h: cells were allowed to attach to the beads with intermittent stirring (1 min of stirring every 20 min), in order to obtain a homogeneous cell distribution. The culture volume was then adjusted to 75 mL by addition of culture

medium, and continuous agitation was set to 45-50 rpm. Twenty four h after inoculation, culture medium was added to obtain the final culture volume (100 mL) and the agitation rate was increased over time (up to 80 rpm).

In all stirred experiments, culture medium was partially replaced (50%) every 3 days. This was done by stopping agitation withdrawing spent medium and refeeding fresh medium immediately after sedimentation of microcarriers/aggregates.

Cell Culture in Bioreactor: A 250 mL bioreactor vessel, designed in house, was used to culture rPSCs in a fully controlled environment. The internal geometry of the vessel is similar to the commercially available spinner vessels but is equipped with pH and pO_2 meters (from Mettler-Toledo, Urdorf, Switzerland) which allow online measuring and control of these parameters. The pH was kept at 7.2 by injection of CO_2 and addition of base (NaOH, 0.2M). The dissolved oxygen concentration was maintained at 30% via surface aeration. The temperature was kept at 37ºC by water recirculation in the vessel jacket controlled by a thermocirculator module. This vessel was adapted to a commercially available bioreactor control unit (B-DCU, B-Braun Biotech International GmbH, Sartorius, Germany) that controls pH, pO_2 and temperature. Data acquisition and process control were performed using MFCS/Win Supervisory Control and Data Acquisition (SCADA) software (B-Braun Biotech International GmbH, Sartorius, Germany).

rPSCs were immobilized in Cytodex 3 microcarries (3 g/L) and directly inoculated in the bioreactor ($1x10^5$ cell/mL) in a working volume of 250 mL. The agitation rate was kept at 50 rpm during the first 24 h and then increased throughout the culture time up to 80 rpm. Culture medium was partially replaced (50%) every 3 days, as described above for the spinner experiments.

2.4. Cell counts and viability

Cell culture samples collected daily were visualized using an inverted microscope with phase contrast (DM IRB, Leica, Germany). Total cell number was determined by counting cell nuclei using a Fuchs-Rosenthal hemacytometer, after digestion with 0.1 M citric acid/1% Triton X-100 (wt/wt) /0.1% crystal violet (wt/v) (Alves et al., 1996).

Cellular viability was assessed by measuring lactate dehydrogenase (LDH) activity from the culture supernatant. LDH activity was determined by following spectrophotometrically (at 340 nm) the rate of oxidation of NADH to NAD^+ coupled with the reduction of pyruvate to lactate. The specific rate of LDH release (q_{LDH}) was calculated for every time interval using the following equation: $q_{LDH} = \Delta LDH/(\Delta t \times \Delta X)$, where ΔLDH is the change in LDH activity over the time period Δt, and ΔXv is the average of total cells during the same time period.

2.5. Growth rate, doubling time and fold increase in cell expansion

Growth rates, doubling times and fold increase parameters were also calculated for all cultures in microcarrier systems. Growth rates (μ) were calculated using a simple first order kinetic model for cell expansion: $dX/dt = \mu X$, where t (day) is the culture time and X (cell) is the value of viable cells for a specific t. μ was estimated using this model applied to the slope of the curves during the exponential phase. Doubling time (t_d) was calculated using the equation $t_d = [Ln(2)]/\mu$. The fold increase in cell expansion (FI) was evaluated based on the ratio X_{MAX}/X_0, where X_{MAX} is the peak cell density (cell/mL) and X_0 is the lowest cell density (cell/mL).

2.6. Metabolite analysis

Glucose (GLC) and lactate (LAC) concentrations in the culture were analyzed using a Bioprofile 100 plus Analyzer (Nova Biomedical, USA). The specific metabolic rates ($q_{Met.}$, mol/(day cell)) were calculated for the two periods between the first two refeeds, using the equation: $q_{Met.} = \Delta_{Met}/(\Delta t\ \Delta X_v)$, where Δ_{Met} is the variation in metabolite concentration during the time period Δt and ΔX_v the average of adherent cells during the same time period. The apparent lactate from glucose yield ($Y_{LAC/GLC}$) was calculated as the ratio between q_{LAC}/q_{GLC}.

2.7. Telomerase activity

The telomerase is responsible for the maintenance of chromosome length and a significant telomerase activity in cells is fundamental for their infinite replication capacity (Masters 2000). Telomerase activity was determined using the Telo TAGGG Telomerase PCR ELISA PLUS kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The assay uses the telomeric repeat amplification protocol (TRAP), in which the telomerase-mediated elongation products are amplified by PCR and detected with an enzyme-linked immunosorbent assay.

2.8. Immunocytochemistry

Cell cultured in microcarriers: Microcarrier cultures were transferred to 1.5 mL tubes and sedimented by gravity. The medium was removed and cells were rinsed with 0.5mM $MgCl_2$ solution in phosphate-buffered saline (PBS), fixed, for 20 min, in 4% (w/v) paraformaldehyde (PFA, Sigma) solution in PBS and permeabilized with Triton X-100 (TX-100, Sigma), 0.3% (w/v) diluted in PBS, during 20 min. After 1 h in blocking solution (bovine serum albumin, BSA, 1% (w/v), and TX-100 0.1% (w/v) in PBS), cells were incubated with primary antibody, for 2 h; washed 3 times with PBS and

incubated with secondary antibody, for 1 h. Microcarriers were transferred to glass slides and covered with a drop of ProLong mounting medium containing DAPI (Molecular Probes). For sample observation, slides were covered with glass coverslips.

Cell Aggregates: After sampling, PBS-washed aggregates were transferred to a tissue protecting compound (Tissue Teck, OCT™ Compound, Sakura) and frozen. Samples were kept at -80ºC until cryosectioning in a Leica Cryostat. For the immunofluorescence assay, 10 μm sections were rehydrated with PBS and fixed in methanol, at -20ºC, during 10 min. After 1 h in a solution of 2% (w/v) BSA and 0.1% (v/v) TX-100 in PBS, the slides were incubated with the primary antibody, for 1 h. After 2 washing steps with PBS, the slides were incubated an additional 45 min with the secondary antibody. At the end, a drop of ProLong mounting medium (Molecular Probes) was added to the slide, that was then covered with a coverslip.

Incubations were done at room temperature. Primary and secondary antibodies used were: monoclonal mouse anti-Nestin (Chemicon) and anti-mouse AlexaFluor 488 (Molecular Probes), respectively, diluted in blocking solution.

All samples were visualized under a fluorescence microscope (DMRB, Leica, Germany).

2.9. Adipocyte differentiation

Cell samples were transferred to static cultures (Tissue culture flasks 75cm^2) and cultured until reaching 100% confluency. At this time, cultures were induced to differentiate into adipocytes by replacing the growth medium with differentiation medium (DMEM containing 10% FBS Gold heat inactivated, 100 U/mL P/S, 10 nM dexamethasone, 0.5 mM

isobutylmethylxantine, 60 μM indomethacin and 100 ng/mL insulin, all from Sigma). Control cultures were run in parallel in medium without additives. The medium was replaced 3 times per week and adipogenesis was observed over a period of 14 days. Differentiation was assessed by morphological characteristics and lipid staining by Oil Red O. Briefly, adipocyte differentiated cultures were washed with PBS and fixed with formalin 10% (Biorad), at room temperature, for 30 min. Cells were then washed twice with sterile water and incubated with 60% isopropanol (Sigma) during 2 min. After that, cultures were stained with fresh solution of Oil red O (1:3 v/v) and counterstained with hematoxylin.

3. RESULTS

3.1. Culture of rPSCs as aggregates

The hypothesis of culturing rPSCs as cell aggregates (Strategy 1) in stirred suspension systems was evaluated using 125 mL spinner vessels. With this strategy, the effect of impeller geometry on cell aggregation and growth was assessed; two impellers configurations were tested: ball (SP-A_B) and paddle (SP-A_P). Initially, the agitation was kept at low rate (50 rpm), in medium supplemented with 15% of FBS, in order to promote cell aggregation (Masters 2000). One day after inoculation, small cell aggregates (70-90 μm) were observed in both cultures by phase contrast microscopy (Figure 2.1A, C). Although cells were able to assemble, single cells were also detected in the culture supernatant. This was particularly observed in SP-A_P (Figure 2.1A), confirming previous reports that show that the ball impeller geometry favors cell aggregation (Moreira et al 1995; Santos et al 2007). From day 1 onwards, SP-A_P aggregates loosed their integrity (Figure 2.1B) and culture viability had a pronounced decrease (data not shown).

In SP-A_B, cell aggregates increased in size, with diameters ranging from 90 to 120 μm upon 3 days of cell cultivation (Figure 2.1D). However, no cell proliferation occurred, suggesting that the increase in size was a result of cell assembling and aggregate clumping. At day 13 (Figure 2.1E), SP-A_B aggregates samples were collected and analyzed for the presence of nestin positive cells, a marker of adult pancreatic stem cells (Kruse 2006), was evaluated by immunofluorescence microscopy in cryosections. Figure 2.1F shows that only a small percentage of cells stained positive for nestin.

3.2. Cell growth and viability of rPSCs cultured in microcarriers

For the second strategy, Cytodex^{TM}1 microcarriers, based on a cross-linked dextran matrix positively charged, and Cytodex^{TM}3, where the dextran matrix is covered with a layer of collagen, were tested for their ability to support the growth of rPSCs in spinner vessels (SP-Cyt1 and SP-Cyt3 experiments). In both studies, cells were inoculated at 1×10^5 cells/mL, corresponding to 5 and 8 cells per microcarrier in SP-Cyt1 and SP-Cyt3, respectively. For all experiments, the microcarrier concentration was 3 g/L, the agitation rate was increased during the culture time from 50 to 80 rpm, and medium replacement was performed every 3 days. Four hours after inoculation, the majority of cells (87.5% of the cell inoculum) were already attached to the microcarriers in SP-Cyt3 (Figure 2.2A), which were able to support cell growth until day 4 (Figure 2.2B). A similar behavior was observed in SP-Cyt1 culture (data not shown), except that, herein, a lower amount of cells adhered to the supports (76.3% of the cell inoculum).

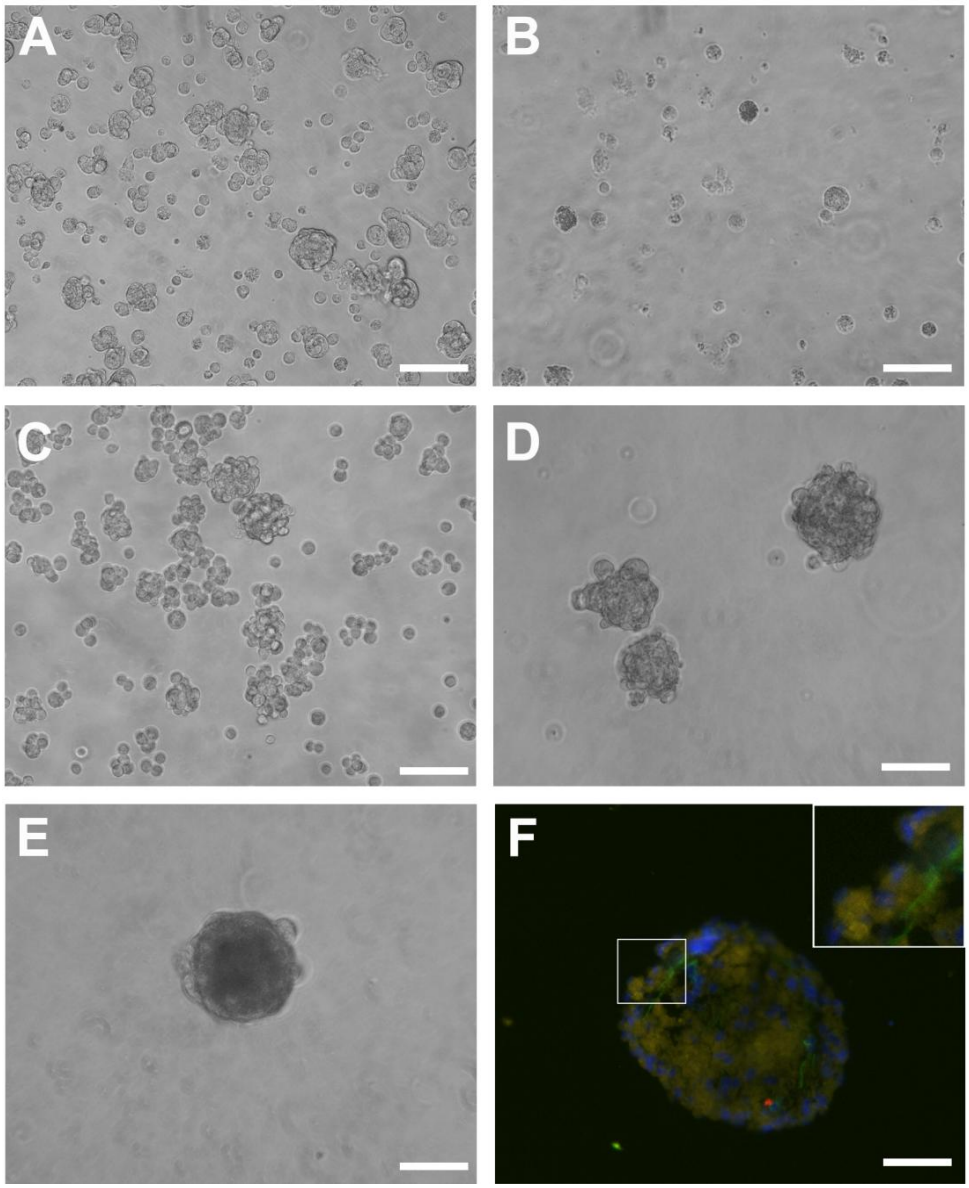

Figure 2.1. Phase contrast photomicrographs of rPSCs cultured as small aggregates in spinner vessels equipped with paddle (A, B) and ball impeller (C, D, E, F). Cells were visualized by day 1 (A, C), day 3 (B, D) and day 13 (E). Immunofluorescence photomicrographs of cell aggregates cryosections at day 13 of ball spinner culture (F). rPSCs were identified by nestin (green) labelling and nuclei were stained with DAPI (blue) Scale bars: (A-E) 100 μm, (F) 50 μm.

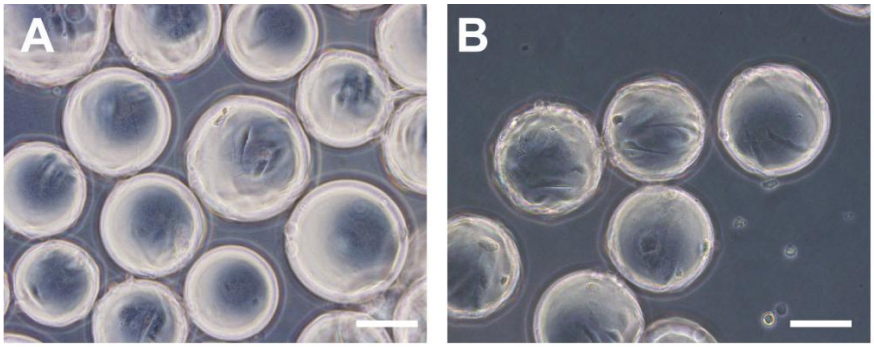

Figure 2.2. Phase contrast photomicrographs of rPSCs cultured in Cytodex 3 microcarriers under stirred suspension conditions. Cells were visualized 4 h after inoculation (A) and by day 4 (B). Scale bar: 100 μm.

Figure 2.3A shows the total cell concentration profile of adherent rPSCs in SP-Cyt1 and SP-Cyt3 experiments. In both cultures, the exponential growth phase lasted until day 4, where the maximum cell concentrations were reached: 1.7 × 10^5 cell/mL and 1.9 × 10^5 cell/mL for SP-Cyt1 and SP-Cyt3, respectively (Figure 2.3A). These values correspond to a 2.2 fold increase in cell concentration for both experiments (Table 2.1), considering the percentage of inoculated cells that attached to the supports.

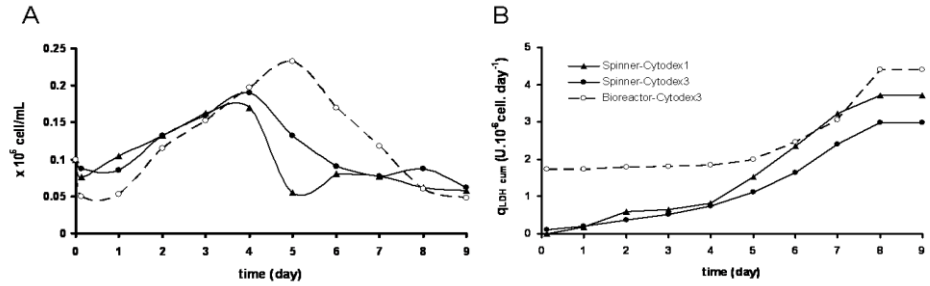

Figure 2.3. rPSCs cultured in microcarriers using stirred suspension systems. (A) Growth curves in terms of total adherent cells per millilitre and (B) viability expressed by the cumulative values of specific LDH release rates.

Growth rates (μ) and doubling times (t_d) were calculated and compared (Table 2.1). A higher growth rate was achieved for SP-Cyt3 (0.26 day^{-1}) than for SP-Cyt1 (0.21 day^{-1}), since the exponential phase period was shorter (3 days) in the first culture. For the same reason, t_d was lower in SP-Cyt3 (2.6 days). Culture viability was analyzed by measuring the cumulative values of specific LDH release rates (q_{LDH}) in the culture supernatant. Figure 2.3B shows that, from day 4 onwards, a significant increase in cumulative q_{LDH} was observed for both experiments (more pronounced in SP-Cyt1). Simultaneously, a decline in total cell number was detected, indicating that cultures had reached the death phase (Figure 2.3A).

Table 2.1. Growth rate (μ), doubling time (t_d) and fold increase (FI) values of rPSCs cultured in cytodex 1 and cytodex 3 microcarriers, using spinner vessels and a 250 mL bioreactor.

	Spinner Cytodex 1	**Spinner Cytodex 3**	**Bioreactor Cytodex 3**
μ (day^{-1})	0.21	0.26	0.35
t_d (day; h)	3.2; 78	2.6; 63	2.0; 47
FI	2.2	2.2	4.7

3.3. Metabolic characterization of rPSCs cultured in microcarrires

Partial medium exchange was performed every 3 days in order to: i) assure supply of nutrients, ii) partially remove metabolic waste products and, more importantly, iii) avoid accumulation of growth/differentiation factors or other secreted molecules capable of inducing rPSC differentiation. Cell metabolism was compared in Cytodex 1 and Cytodex 3 microcarriers cultures to ensure that nutrient consumption and waste production were not limiting rPSC growth. Measurement of metabolite concentrations in the

medium (namely glucose and lactate) were performed daily and are presented in Figure 2.4. The results showed that for both cultures and up to 9 days, there was no depletion of glucose levels. Concerning metabolic product, the levels of lactate never reached 20 mM, except in SP-Cyt1 at the last 2 days of cultivation (Figure 2.4).

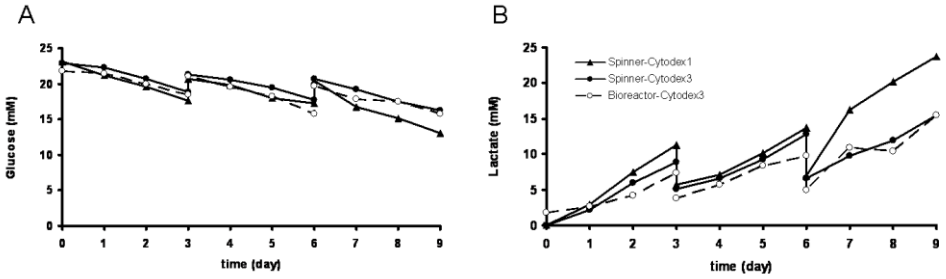

Figure 2.4. Glucose uptake and lactate release of rPSC cultured in Cytodex 1 and Cytodex 3 microcarriers, using spinner vessels and a 250 mL bioreactor. Metabolite concentrations are shown over 9 days of culture.

In order to better characterize and compare the cell metabolism pattern of the two microcarrier cultures, the specific rates of nutrient consumption (q_{GLC}) and metabolite production (q_{LAC}) were calculated. Two phases were considered: one until the first reefed (Phase 1, from day 0 to day 3) and the other between the first and second refeeds (Phase 2, from day 3 to day 6) (Table 2.2).

The values obtained for both cultures were similar. Overall, the specific consumption and production rates were higher within Phase 1, which is consistent with the high activity of cells at the beginning of the exponential phase in cell growth. In Phase 2, a decrease on all specific rates (q_{GLC} and q_{LAC}) was detected; at this time, cells were at the end of the exponential phase and entering into the death period, usually associated with a slowdown of their metabolic activity. The yields of lactate production from glucose consumption ($Y_{LAC/GLC}$) were also calculated in order to evaluate

the efficiency of glucose metabolism (Table 2.2). The results showed that overall, $Y_{LAC/GLC}$ were higher than 2, suggesting that glucose was totally converted to lactate via glycolysis and that other carbon sources are eventually being canalized to generate lactate. These findings indicate the occurrence of oxygen limitation in both spinner experiments.

Table 2.2. Metabolic characterization of rPSCs cultured in Cytodex 1 and Cytodex 3 microcarriers, using spinner vessels and a 250 mL bioreactor. The specific rates of glucose consumption (q_{GLC}) and lactate production (q_{LAC}) were calculated from day 0 to day 3 (Phase 1) and from day 3 to day 6 (Phase 2). The apparent lactate from glucose ($Y_{LAC/GLC}$) yields was calculated for the two phases.

	Spinner Cytodex 1		Spinner Cytodex 3		Bioreactor Cytodex 3	
	Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
q_{GLC} $\mu mol/(10^6 cells.days)$	15.1	10.6	13.0	8.0	12.7	9.5
q_{LAC} $\mu mol/(10^6 cells.days)$	32.5	23.5	29.0	17.9	20.3	11.4
$Y_{LAC/GLC}$	2.1	2.2	2.2	2.2	1.6	1.2

3.4. rPSCs expansion in a fully controlled bioreactor

Since Cytodex 3 microcarriers have been shown to preferentially support PSCs growth on stirred suspension systems (best cell adherence and faster cell growth), the next challenge was to scale-up and control rPSCs growth by culturing cells in a 250 mL controlled bioreactor (BR-Cyt3). In this vessel, cells were cultured in the same conditions used for SP-Cyt3 (agitation rate- 50 to 80 rpm, inoculum concentration- 1 x 10^5 cell/mL, bead concentration- 3g/L), except that, herein, cells previously attached to Cytodex 3 microcarriers were directly inoculated in the bioreactor. Furthermore, cultivation was performed in a fully controlled environment

(temperature-37ºC, pH-7.2, and pO_2-30%), in order to overcome the oxygen limitation observed in the spinner culture (described above).

Total cell concentrations of adherent cells are represented in Figure 2.3A, as well as the cumulative q_{LDH} values achieved throughout culture time in Figure 2.3B. The first 4 h were characterized by a pronounced decrease in cell number; 50% of the total starting cells were detached from the beads. Simultaneously, q_{LDH} was significantly higher at this time point than in spinner culture, confirming that increased cell lysis was taking place. Phase contrast photomicrograph showed that a smaller amount of cells remained attached to microcarriers in BR-Cyt3 than in SP-Cyt3 (Figures 2.5A and 2.2A, respectively), confirming that the inoculation process was somehow aggressive for the cells. After 24 h, cells started to divide with a growth pattern similar to that obtained for SP-Cyt3, except that the exponential phase was extended until day 5 (Figure 2.3A). At this time, a high percentage of supports were totally covered by rPSC (Figure 2.5B); cell concentration reached the maximum value (2.3×10^5 cell/mL), yielding a two times higher fold increase in cell concentration than in SP-Cyt3 (4.6 versus 2.2) (Table 2.1). From day 5 onwards, cells reached the death phase and detached from the beads (Figure 2.5C), resulting in increasing q_{LDH} levels (Figure 2.3B). Growth characteristics were also compared for both culture systems (Table 2.1). The results obtained showed the apparent growth rate was higher (0.35 day^{-1}) and the doubling time was lower (47 h or 2 days) in BR-Cyt3 than in SP-Cyt3, confirming that cell growth was faster in the bioreactor culture. Overall these results can be justified by the metabolic performance of cells when cultured in a fully controlled environment. Neither depletion of nutrients nor accumulation of inhibitory waste concentrations was detected over culture time (Figure 2.4). The specific rates of metabolites consumption and production were estimated (Table 2.2). Furthermore no changes were detected in glucose

consumptions, while the levels of lactate production were significantly lower, resulting in yields of $Y_{LAC/GLC}$ lower than 2 (1.6 and 1.2 for phase 1 and phase 2, respectively); due to the higher oxygen availability to the cells, there was a more efficient use of glucose, thus resulting in a higher production of biomass

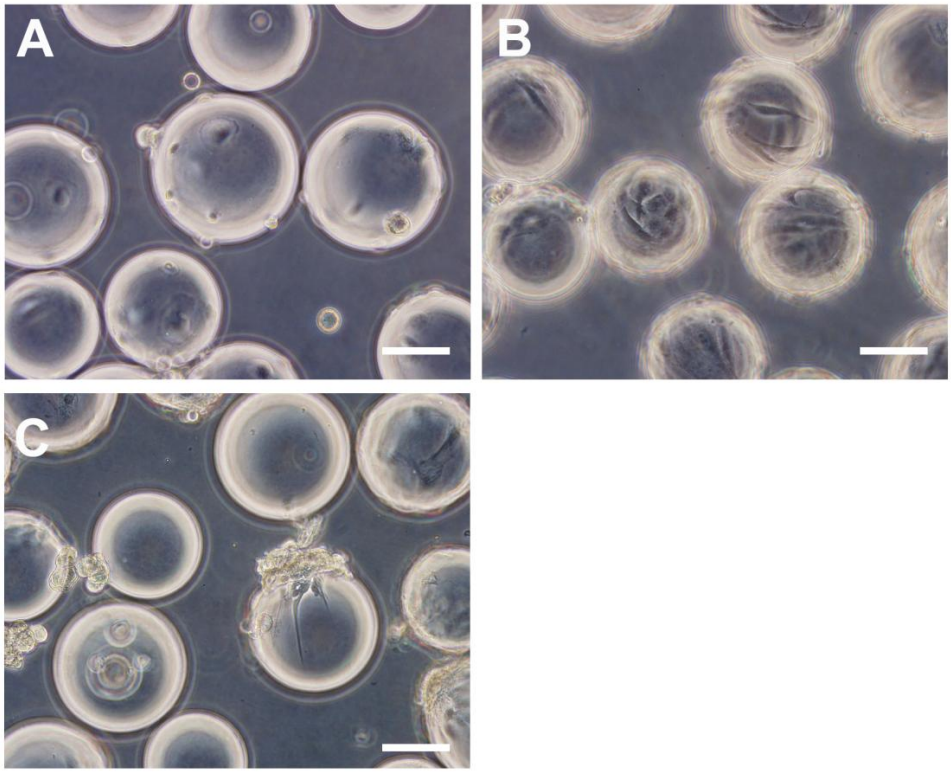

Figure 2.5. Phase contrast photomicrographs of rPSCs cultured in Cytodex 3 microcarriers in a 250 mL bioreactor. Cells were visualized after inoculation (A), by day 4 (B) and at the end of cell cultivation, i.e., by day 9 (C). Scale bar: 100 μm.

3.5. Characterization of rPSCs expanded in microcarriers

The distribution of rPSCs on the microcarriers surface was visualized by nestin staining using immunofluorescence microscopy. In the experiments

using the Cytodex supports, adherent cells, identified by nuclear DAPI staining, stained positive for nestin (Figure 2.6A).

To evaluate the efficiency of each microcarrier culture system in expanding rPSCs, all populations were characterized in terms of self-renewal capacity and differentiation potential; this was done by evaluating telomerase activity and inducing adipocyte differentiation, respectively. The telomerase activity was analyzed for rPSCs derived from both static and microcarrier cultures (Figure 2.6C). In all cases, the absorbance readings of samples (A_{450nm}-A_{690nm}) were higher than two-fold the value of the respective negative control (heat-treated samples), confirming that all samples were telomerase positive. In microcarrier cultures, the levels of telomerase activity were similar to the control, showing that expanded PSCs maintained the capacity to proliferate extensively *in vitro*. To examine the differentiation potential, rPSCs were cultured in adipocyte differentiation medium for 2 weeks. The presence of lipid deposits stained with Oil Red O (Figures 2.6B) demonstrated that these cells were able to differentiate into adipocytes. In conclusion, all these findings demonstrate that after expansion in microcarriers, cells maintained the expression of rPSC marker, their self-renewal ability and differentiation potential, confirming that they retained their stem cell behavior.

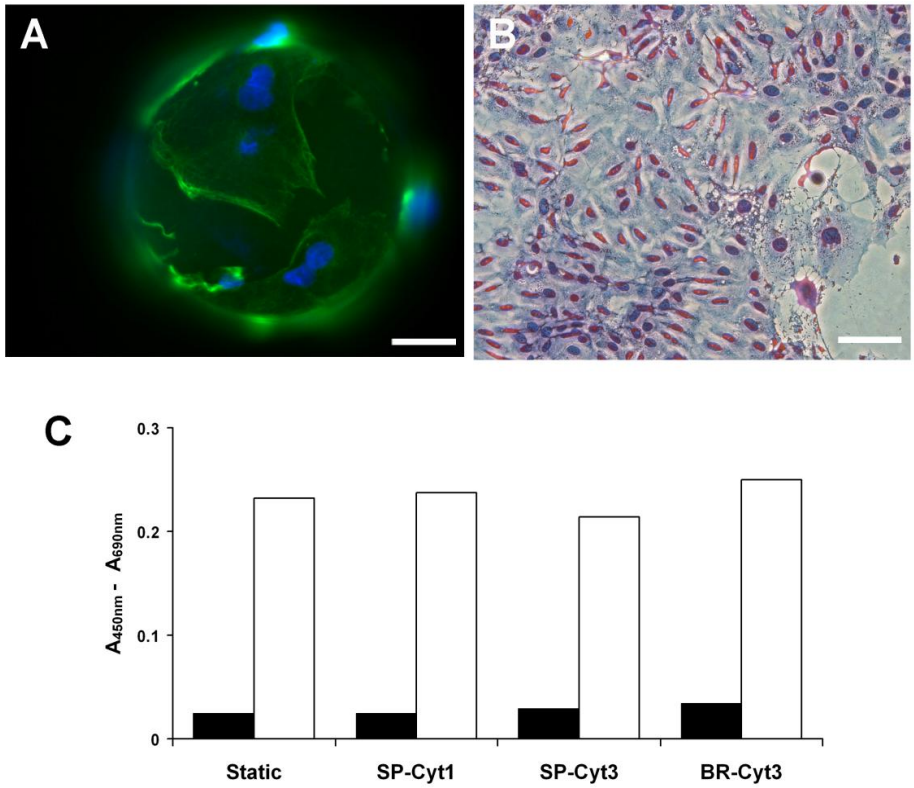

Figure 2.6. Characterization of rPSCs cultured in Cytodex 3 microcarriers. By day 7, rPSCs growing adherent to the carriers were identified by nestin labelling (green), using immunofluorescence microscopy (A). Adipogenic differentiation was evaluated by lipid staining by Oil Red O (B). Scale bars: (A) 50 μm and (B) 100 μm. Telomerase activity of rPSCs was analyzed by day 7 of culture using a Telo TAGGG Telomerase PCR ELISA ^{PLUS} kit (C). The white bars show the results obtained for the rPSCs expanded in static (Static) and stirred (SP-Cyt1, SP-Cyt3 and BR-Cyt3) conditions. Negative controls (black bars) represent telomerase activity following inactivation by heat.

4. DISCUSSION

The first purpose of stem cell cultivation is to expand cells while maintaining their pluripotent potential, in order to produce large numbers of highly pure populations, adequate for controlled differentiation. In the present study, we

successfully developed an efficient strategy for the expansion of rPSC cultures in Cytodex 3 microcarriers using fully controlled bioreactors.

A vast range of microcarriers is currently available, employing different materials and surface types, in order to allow the culture of different anchorage-dependent cell types in stirred conditions. This strategy has been widely used by our group in the development of scalable bioprocesses to grow mammalian cells in suspension bioreactors (Alves et al., 1996; Sa Santos et al., 2005). In this work, the two microcarriers tested (Cytodex 1 and Cytodex 3), provided a suitable matrix for the expansion of PSCs under stirred conditions. A more efficient and robust cell adherence and proliferation was obtained using Cytodex 3 beads (Figure 2.3), which may be explained by the better adhesion of rPSCs to the collagen layer that covers the surface of the carriers. Cytodex 3 is specially designed for culturing sensitive cells and has been reported to be suitable for culture of primary brain cells (Santos et al., 2005), as well as for the expansion of mouse ESCs in suspension stirred systems (Fok and Zandstra, 2005; Abranches et al. 2007).

Expanding rPSCs adherent to Cytodex 3 microcarriers resulted in stem cell population that retained their characteristics: rPSC marker, self-renewal ability and differentiation potential (Figure 2.6), proving to be of great advantage over culturing the cells as aggregates, where cells clumped together, did not proliferate and lost their nestin labeling (Figure 2.1). Although cell aggregates have been used in previous reports to expand undifferentiated murine embryonic stem cells (Cornier et al., 2007; zur Nieden et al., 2007), neural stem cells (Gilbertson et al., 2006) and carcinoma stem cells (Youn et al 2006), this approach revealed to be unfeasible for culturing PSCs (Figure 2.1). These differences in cell behavior may reflect the distinct tissue origins, as rPSCs are pluripotent adult stem cells derived from pancreas (Kruse et al 2004, 2006). Our

results suggest that rPSCs are anchorage-dependent and that adherence plays a role in the control of cell fate decisions.

The potential of bioreactor technology was combined with the efficient Cytodex 3 strategy, resulting in a robust bioprocess for the expansion of PSCs. Under controlled environment (pH, pO_2 and temperature), cell growth was more efficient, as shown by faster doubling time, higher growth rate and higher fold increase, when compared to spinner cultures (Table 2.1). This improvement may be explained by the higher availability of oxygen (supplied by the bioreactor controller) to the cells. Comparison of apparent lactate from glucose yields obtained in stirred bioreactor and spinner vessels ($Y_{LAC/GLC}$ = 1.6-1.2 and 2.2, respectively, Table 2.2) indicates that cells metabolized glucose more efficiently in the bioreactor, with lower production of lactate. Thus, the oxygen limitation observed in the spinners was successfully overcome in the bioreactor experimental setup, leading to an increased level of cell proliferation.

The fold increase in cell concentration obtained in the bioreactor was not as high as those achieved for mESC cultured in Cytodex-3 microcarriers in spinner vessels (Fernandes et al, 2007) and neonatal porcine pancreatic cells cultured as spherical-islets (Chawla et al, 2006). PSCs are fusiform cells, with sizes up to 50 -100 µm, which limited the cell expansion ratio in microcarriers to the approximately 5-fold increase obtained in this study. Thus, in order to obtain higher expansion ratios, serial passaging with addition of fresh microcarriers can be incorporated. Moreover, alternative strategies, such as perfusion or fed-batch culture mode allowing the microcarrier feeding may be considered.

In summary, this controlled and robust culture system for expansion of multipotent adult rPSCs, using stirred bioreactors, has proven to be a strong starting point for the development of novel technologies for cell therapy.

5. ACKNOWLEDGMENTS

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CHAPTER 3

NOVEL STRATEGY FOR NEURONAL DIFFERENTIATION OF HUMAN STEM CELLS

This chapter was based on the following manuscript:

Serra, M., Leite, S.B., Brito, C., Costa, J., Carrondo, M.J.T., Alves, P.M., 2007. Novel culture strategy for human stem cell expansion and neuronal differentiation. J Neurosc Res 85(16), 3557-3566.

ABSTRACT

Embryonal carcinoma (EC) stem cells derived from germ cell tumours closely resemble embryonic stem (ES) cells and are valuable tools for the study of embryogenesis. Human pluripotent NT2 cell line, derived from a teratocarcinoma, can be induced to differentiate into neurons (NT2-N) after retinoic acid treatment. To realize the full potential of stem cells, *in vitro* methods for stem cell proliferation and differentiation constitute a key challenge.

Herein, a novel culture strategy for NT2 neuronal differentiation was developed to expand NT2-N neurons, reduce the time required for the differentiation process and increase the final yields of NT2-N neurons. NT2 cells were cultured as 3D cell aggregates ("neurospheres") in the presence of retinoic acid, using small scale stirred bioreactors; it was possible to obtain a homogeneous neurosphere population, which can be transferred for further neuronal selection into coated surfaces. This culturing strategy yields higher amounts of NT2-N neurons with increased purity, as compared with those routinely obtained for static cultures. Moreover, mechanical and enzymatic methods for neurosphere dissociation were evaluated in their ability to recover neurons, trypsin digestion yielding the best results. Nevertheless, highest recoveries were obtained when neurospheres were collected directly to treated surfaces without dissociation steps.

This novel culture strategy allows improving drastically the neuronal differentiation efficiency of NT2 cells as a 4-fold increase was obtained, reducing simultaneously the time needed for the differentiation process. The culture method described herein ensures efficient, reproducible and scaleable ES cell proliferation and differentiation, contributing for the usefulness of stem cells bioengineering.

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1. INTRODUCTION

Human embryonic stem cells (hESCs) have been the focus of intense research since the first successful isolation of human inner cell mass (ICM) in 1994 (Bongso et al., 1994) and the establishment of the first hESC line nearly 4 years later (Thomson et al., 1998). These cells can be propagated in culture for extended periods and can differentiate into all somatic cell types (pluripotent cells), thus holding enormous prospect for the development of novel approaches for improving processes in cell therapy, tissue engineering, drug discovery and *in vitro* toxicology (Jones and Thomson, 2000; Davila et al., 2004; McNeish 2004). However, assessing and characterizing hESC lines can be difficult, time consuming and expensive due to demanding cell culture procedures and requirements under existing patents. To overcome this, hESC model systems are very valuable for predicting cellular behaviour and elucidating mechanisms involved in self-renewal and differentiation. Within this, several groups have proposed using embryonal carcinoma (EC) cell lines based on earlier observations that germ cells are pluripotent (Pal and Ravindran, 2006) and share characteristics with ESCs (Andrews, 2002).

Pluripotent EC cells are derived from tumours known as teratocarcinomas that are understood to arise from transformed germ cells (Przyborski et al., 2004). It is generally accepted that EC cells closely resemble ES cells and are often considered to be the malignant counterparts of ES cells (Andrews et al., 2001). In fact, EC stem cell lines provide a useful alternative to embryos for the study of mammalian cell differentiation (Stewart et al., 2003). More specifically, the well-established NTera-2/cl.D1 (NT2) lineage, which has been derived from a human testicular cancer, offers a convenient and robust model for studying the commitment of human EC stem cells to the neural lineage and their subsequent differentiation into neurons (Andrews, 1984). Upon treatment with retinoic acid (RA), the NT2

cells can be induced to differentiate into postmitotic neurons (NT2-N) that express many neuronal markers (Pleasure et al., 1992). Using specific culture conditions these NT2-N neurons form functional synapses (Hartley et al., 1999) and express a variety of neurotransmitter phenotypes (Pleasure et al., 1993; Yoshioka et al., 1997; Guillemain et al, 2000). This cell line has also been used in several transplantation studies including engraftments in experimental animals (Ferrari et al, 2000; Watson et al., 2003) and in human patients (Kondziolka et al., 2001).

Although techniques have been developed to produce and purify NT2-N neurons from other contaminating cell types, these methods are laborious, yield relatively few neurons *per* culture, and are thus time consuming. Therefore, the last 2 years have witnessed an increase in experiments aimed at improving culture systems for enhanced neuronal productivity and reduced differentiation time (Durand et al., 2003; Horroks et al., 2003). These studies are focused on the cultivation of free floating cell spheres (neurospheres) under suspension conditions, using non-adherent Petri dishes. However, these static culture systems have several limitations that affect culture productivity, scalability and specially reproducibility. To overcome these limitations novel culture systems for proliferation and differentiation of NT2 cells are needed. So far, due to their characteristics and wide use, stirred culture systems, including fully controlled bioreactors, appear as promising candidates, as they are hydrodynamically well characterized, easy to scale-up, enable better homogeneity of cultures, reproducible experimental conditions and allow easy sampling. Within the field of stem cell expansion and differentiation several reports have been published using stirred tank bioreactors (Collins et al., 1998a; Collins et al., 1998b; Sen et al., 2002; Youn et al., 2005; Cameron et al., 2006). However, these are previous approaches and more innovative strategies need to be explored. Up to now, the biggest restriction is still the low efficiency of the

stem cell differentiation process and this limitation is very critical when aiming towards scaleable protocols.

The present work describes a novel culture system that enables the improvement of expansion and differentiation of NT2 cells. Herein, for the first time, a strategy was established to obtain neuronal differentiated from non-neuronal EC cells using stirred suspension conditions. Thus, culturing the NT2 cells as 3D aggregates (neurospheres) permitted increased yields of differentiated NT2-N neurons to be obtained in a shorter process time. The improvement of the culture system described here opens new perspectives for hESC technology field, by allowing promising strategies for stem cell proliferation and differentiation, a bottleneck for the expansion of this science and technology area.

2. MATERIAL AND METHODS

2.1. Cell culture

Undifferentiated NT2 cells were routinely cultivated in standard tissue culture flasks (T75 culture flasks from Nunc) and maintained in OptiMEM medium (Gibco) supplemented with 5% (v/v) of fetal bovine serum (FBS, Hyclone) and 100 U/mL penicillin- streptomycin (P/S, Gibco).

2. 2. Systems for NT2 neuronal differentiation

In order to evaluate the effectiveness of the culture strategy developed herein (stirred conditions) protocols for culturing and differentiation NT2 cells in static conditions reported in the literature were also performed. A brief description of both methodologies is summarized in Table 3.1.

Table 3.1. Comparison of neuronal differentiation protocols in static and stirred culture conditions for NT2 cells.

Static Culture Protocol (Pleasure et al., 1992)		Stirred Culture Protocol	
	NT2 proliferation and Differentiation		**STEP 1 – NT2 Proliferation and Differentiation**
-	Inoculation of precursor NT2 cells in Tflasks	-	Inoculation of precursor NT2 cells in Spinner vessels
1 day	Culture of NT2 cells without RA	2 days	Culture of NT2 cells without RA – Growth Period
34 days	RA treatments	14 days	RA treatments – Differentiation Period
	Medium replacement (100% - 2 times per week)		Medium replacement (50%, 3 times per week)
-	**Replate 1 – Replating of cells at lower density**		**STEP 2 – Neurosphere Harvesting**
12 days	Mitotic inhibitor treatment	7 days	Mitotic inhibitor treatment
	Medium replacement (100% - 2 times per week)		Medium replacement (100% - 2 times per week)
	Replate 3 – Selecting NT2-N neurons		**STEP 3 – Selecting NT2-N neurons**
0-5 days	Cultivation in conditioned medium	0-5 days	Cultivation in conditioned medium
Total 47-53 days	Harvesting of NT2-N neurons	Total 23-28 days	Harvesting of NT2-N neurons

2.2.1. Experiments in static conditions

Control experiments of NT2 differentiation were done according to the procedure described in Pleasure et al 1992. Briefly, NT2 precursor cells were cultured in standard tissue culture flasks (T75 culture flasks from Nunc) and differentiated in Dulbecco's Modified Eagle's Medium (DMEM)-High Glucose (HG) (Gibco) with 10% (v/v) FBS, 100 U/mL P/S and 10 µM retinoic acid (RA, Sigma) for five weeks into postmitotic NT2-N neurons. This cell culture was then trypsinized and replated at a lower density (total cells collected from one T75 culture were equally divided by two T175) and cultured in DMEM-HG with 5% (v/v) FBS containing 100 U/mL of P/S and mitotic inhibitors (1 µM cytosine arabinoside, AraC, 10 µM fluorodeoxyuridine, Fudr, and 10 µM uridine, Urd) (Sigma) for 12 days in order to obtain a NT2-N culture with maximum purity. After this period of culture in T175 culture flasks, NT2-N neurons were selectively trypsinized using a 1:3 diluted trypsin solution (Trypsin-EDTA 1X, liquid 0.05% Trypsin,

Gibco) in DMEM-HG medium. Subsequently, the cells were counted and transferred to plates/slides coated with PDL and MG (PDL-MG) for further experiments, including culture characterization using immunocytochemistry tools.

2.2.2. Experiments in stirred suspension conditions

This protocol was designed into three sequential steps where only the first one is performed in stirred suspension conditions.

Step 1 - NT2 proliferation and differentiation in spinner vessels: NT2 cells were inoculated in a 125 ml spinner vessel (from Wheaton, Techne, NJ) equipped with ball impeller, at a density of $5x10^5$ cell/ml. Throughout this step, cells were cultured in stirred suspension conditions for 16 days.

Growth Period: During the first two days, undifferentiated $NT2^-$ cells were cultured in 80 ml of DMEM-HG medium (Invitrogen)) supplemented with 10% (v/v) FBS (Hyclone) and 100 U/mL of P/S (Invitrogen)).

Differentiation Period: From the third day onwards every 2-3 days (i.e, three times per week), retinoic acid (RA, Sigma) was added to the medium to yield a final concentration of 10 μM in 100 ml of culture volume. Since sedimentation of neurospheres (similar to earlier published work described in Moreira et al, 1994) was too slow, yielding necrotic centers and dramatic loss of viability, another strategy was attempt; transferring half of culture supernatant to centrifuge tubes. After centrifugation at 200 g for 10 min, the collected cells/aggregates were ressuspended in new culture medium containing RA. Then, the cell suspension obtained was added to the remaining cell suspension in the spinner vessel to yield a final volume of 100 mL. The agitation rate was increased during cultivation in order to avoid aggregate clumping and to control neurosphere size (Day 1 to Day 9 – 60 rpm, Day 10 to Day 14 – 80 rpm, Day 15 upwards – 100 rpm).

Step 2 – Neurosphere Harvesting: After 16 days of culture, corresponding to 2 weeks of differentiation time, the NT2 neurospheres were collected. After that, both intact and dissociated neurospheres were plated (cell density 1-3x10^5cells/cm^2) on T75 culture flasks (Nunc) coated with poly-D-lysine (PDL, Sigma) and Matrigel (MG, Becton-Dickinson), and cultured under mitotic inhibitory conditions: DMEM-HG (Invitrogen)) supplemented with 5% (v/v) FBS (Hyclone), 100 U/mL of P/S (Invitrogen)), 1 μM Ara C, 10 μM FudR and 10 μM Urd (Sigma); the culture medium was partially (50%) replaced every 2 days.

Step 3 – Selecting NT2-N neurons: After 7 days of culture in T75 culture flasks, NT2-N neurons were selectively trypsinized using a 1:3 diluted trypsin solution (Trypsin-EDTA 1X, liquid 0.05% Trypsin, Invitrogen)) in DMEM-HG medium. Subsequently, the cells were counted and transferred to plates/slides coated with PDL and MG (PDL-MG) for further experiments, including culture characterization using immunocytochemistry tools.

2.3. Aggregates dissociation test

Five protocols were compared for the dissociation of NT2 neurospheres obtained after cultivating aggregates in spinner vessel for 16 days, including mechanical and enzymatic methods. The same cell concentration was used for each dissociation protocol. In the mechanical assay, NT2 aggregates were dissociated using a fire narrowed Pasteur pipette. In the enzymatic protocols, neurospheres were disrupted using four different solutions: TrypLe Select (Invitrogen), Trypsin-EDTA (1X) (liquid 0.05% Trypsin, Invitrogen), Accutase (Chemicon) and Accumax (Chemicon). For all assays a similar procedure was used: 1 mL of cell suspension was collected for each protocol and after centrifugation (200 g, 10 min) the neurospheres were incubated with 200 μL of dissociation enzyme at 37ºC during 2 min. Then, 800 μL of serum supplemented medium were added to

each sample. In all procedures cell density and viability were evaluated after dissociation. Cell density was assessed using a hemacytometer and viability was determined using the standard Trypan Blue exclusion test. The experiments were performed in triplicate.

The different cell suspensions were then plated on 6-well plates (Nunc) precoated with PDL-MG and cells were cultured for up to 7 days under mitotic inhibitory conditions. All cultures were monitored daily using an inverted microscope (Leica, DM IRB).

2.4. Aggregate size

The aggregate size in each culture sample was measured using a micrometer coupled to an inverted microscope (Leica, DM IRB). The average diameter was calculated by measurement of two perpendicular diameters of a minimum of 15 aggregates. Aggregates less than 20 μm in diameter (generally single cells or doublets) were not considered.

2.5. Immunofluorescence microscopy

Cell culture slides and cell aggregates: Aggregates and cell culture grown on glass coverslips were rinsed in phosphate-buffered saline (PBS) with 0.5mM $MgCl_2$ and fixed 20min in 4% (w/m) paraformaldehyde (PFA) solution in PBS with 4% (w/v) saccarose. After fixation cells were washed 2 times with PBS and then incubated with permeabilization solution, TritonX-100, 0.3% (w/v) diluted in PBS, during 20 min. Consequently, cells were washed again with PBS and kept in blocking solution composed of bovine serum albumine (BSA) 1% (w/v), and TritonX-100 0.1% (w/v) in PBS, for 1 h. Cells were then incubated with primary antibodies diluted in PBS containing 0.1% (w/v) of TritonX-100, for 2 h at room temperature; the coverslips were washed 3 times with PBS and overlayed with secondary antibodies for 1 h at room temperature. After 3 washes with PBS, samples

were mounted in ProLong medium, supplemented with DAPI (Molecular Probes), for nucleus staining. Primary antibodies and dilutions used were: anti-Nestin (Nestin, 1:200, Chemicon), anti-Tubulin beta III isoform (β-TubIII, 1:10, Chemicon), anti-Neurofilament, light chain (NF-L, 1:200, Chemicon), anti-Neurofilament, heavy chain (NF200, 1:200, Chemicon), anti- Microtubule associated protein-2 (MAP2a&b, 1:100, Chemicon), anti-Glial fibrillary acidic protein (GFAP, 1:200, Chemicon) and anti-Oligodendrocyte marker O4 (O4, 1:200, Chemicon). The secondary antibodies and dilutions used were: anti-mouse Alexa 488 (1:200) and anti-rabbit Alexa 594 (1:500) from Molecular Probes. Cells were visualized using a fluorescence microscope (Leica DMRB).

3. RESULTS

3.1. NT2 neurospheres cultured in spinner vessel

The ability to consistently and reproducibly grow large numbers of human neural cells *in vitro* is often limited by complex and demanding protocols. Since NT2 cells have been reported to successfully proliferate and differentiate on non-adhesive substrates as cell clusters (Durand et al., 2003; Horroks et al., 2003), herein the challenge was to cultivate the NT2 neurospheres in scalable and robust culture systems.

Therefore, NT2 precursor cells were inoculated in a spinner vessel, at a density of $5x10^5$ cell/mL. During the following 2 days of culture (Growth Period) the agitation rate was set up to low values in order to promote NT2 cell aggregation. Under these conditions the NT2 precursor cells proliferated with a two-fold increase in cell concentration (results not shown) and successfully aggregated into small cell aggregates ranging from 50 to 100 μm (average diameter 60.1 ± 7.6 μm) (Figure 3.1A).

Neuronal differentiation was induced by addition of RA two days after cell inoculation (Differentiation Period). Initially such aggregates had a ragged appearance (Figure 3.1B) but, as differentiation progressed their size diameter increased (ranging from 100 to 300μm – average diameter 160.0 ± 78.6μm) and their structure stabilized forming solid and spherical 3D aggregates- neurospheres (Figure 3.1C, D). Here, an essential parameter that needs monitoring control is the aggregate/neurosphere size, which should be minimized in order to avoid detrimental diffusion gradients and/or decrease the differentiation potential of the culture. As reported previously (Moreira et al., 1994; Kallos et al., 2003), the agitation rate in suspension systems should be sufficient to control the diameter of neurospheres. Therefore, in this study, the rate of agitation was increased during culture time in order to ensure a homogenous neurosphere culture and avoid necrotic and apoptotic cells within the neurospheres.

After two weeks of RA treatments neurospheres were characterized in terms of cell composition using immunofluorescence microscopy. Figure 3.1E shows that, at this time, neurospheres were composed by both nestin positive cells (undifferentiated stem cells and neural progenitor cells) and β–tubIII positive cells (NT2-N neurons).

3.2. Neurospheres harvesting

At day 16, neurospheres were collected and two different strategies were tested in order to evaluate the best method to recover the NT2-N neurons. On both strategies the cells were seeded into PDL-MG coated surfaces and cultured under mitotic inhibitory conditions for one week.

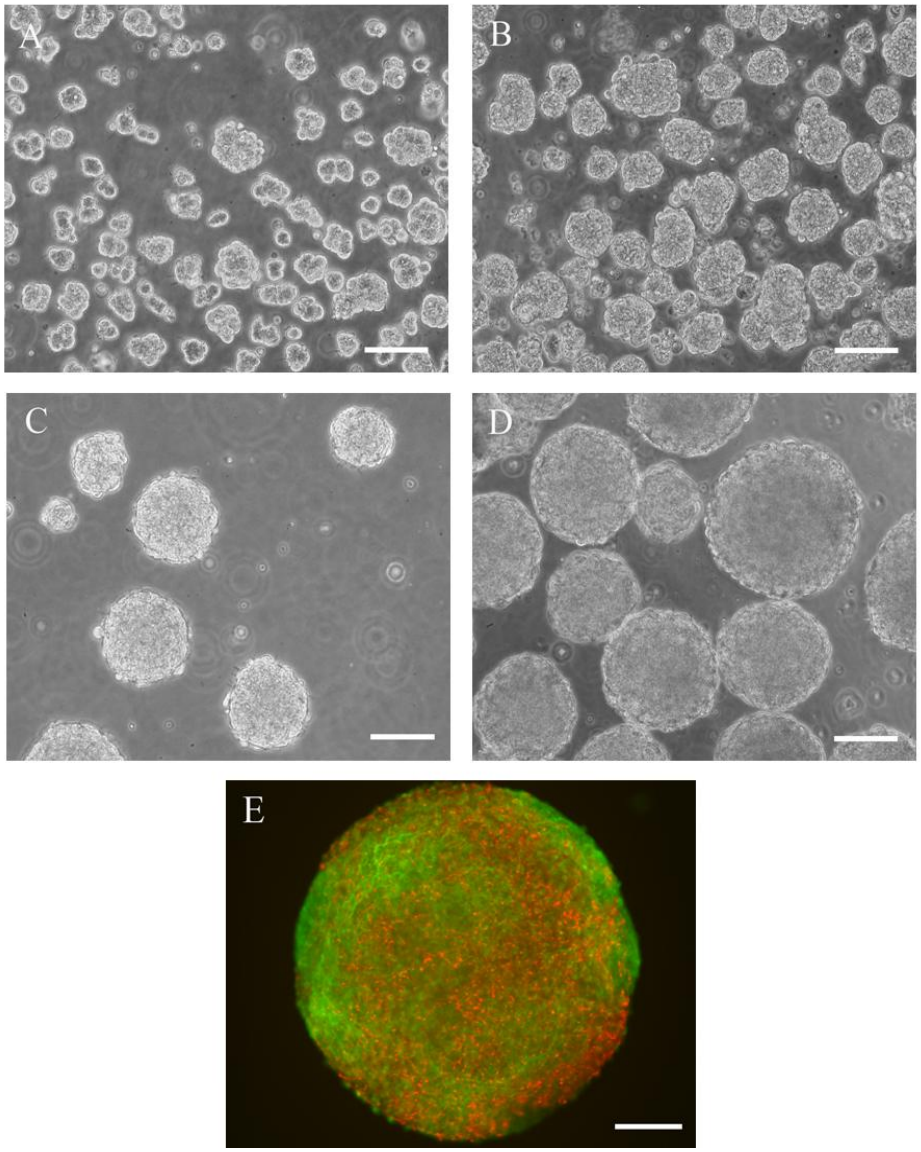

Figure 3.1. Phase contrast photographs of NT2 cells cultured in stirred conditions (spinner). Small cell aggregates (≈ 50 μm) were observed at day 2 (A). Afterwards these neurospheres grew in number (B- day 5) and in size (C- day 10), yielding an average diameter of approximately 300 μm at day 15 (D). (E) Immunofluorescence image of NT2 neurospheres collected at day 16: double labelling with b-TubIII (green) and nestin (red).Scale bar: 100μm (A,B,C,D); 50μm (E).

On the first strategy, neurospheres were dissociated using five different protocols (including enzymatic and mechanical methods). In order to investigate and select the best procedure, these results were compared in terms of final single cell concentration and viability after each specific dissociation procedure. As shown in Figure 3.2, enzymatic methods led to higher cell viabilities (> 90% in all protocols) than the mechanical procedure. In addition, higher amounts of cell debris were present in mechanical dissociated cultures. These findings suggest that the mechanical procedure is very aggressive for NT2 neurosphere dissociation. When the enzymatic dissociation protocols were compared for final single cell concentration, the highest value was observed for samples dissociated with Trypsin, which was also the method that enhanced better neurosphere dissociation, when monitored by microscopic inspection (Figure 3.3, Day 0). The remaining enzymatic dissociation assays (TrypLE Select, Accutase and Accumax) were less efficient as several cell aggregates were detected in each cell suspension following the dissociation procedure (Figure 3.3, Day 0). Moreover, the values of final cell concentration were lower when compared to the samples dissociated with Trypsin (Figure 3.2).

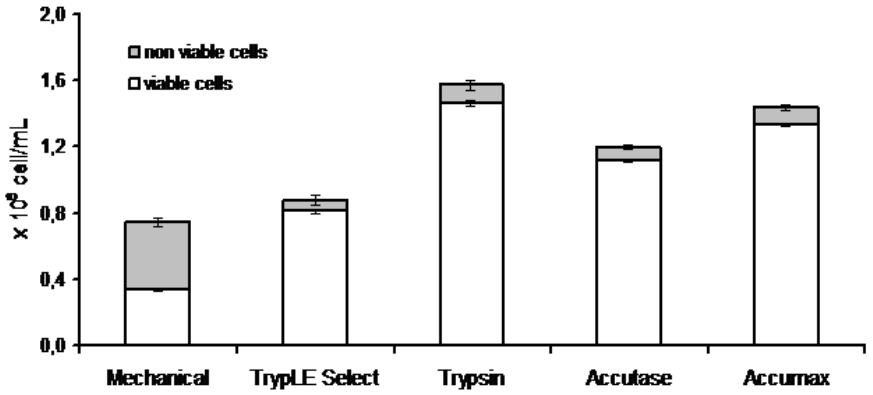

Figure 3.2. Effect of neurosphere dissociation protocol in concentration of viable (white bars) and non viable (grey bars) cells.

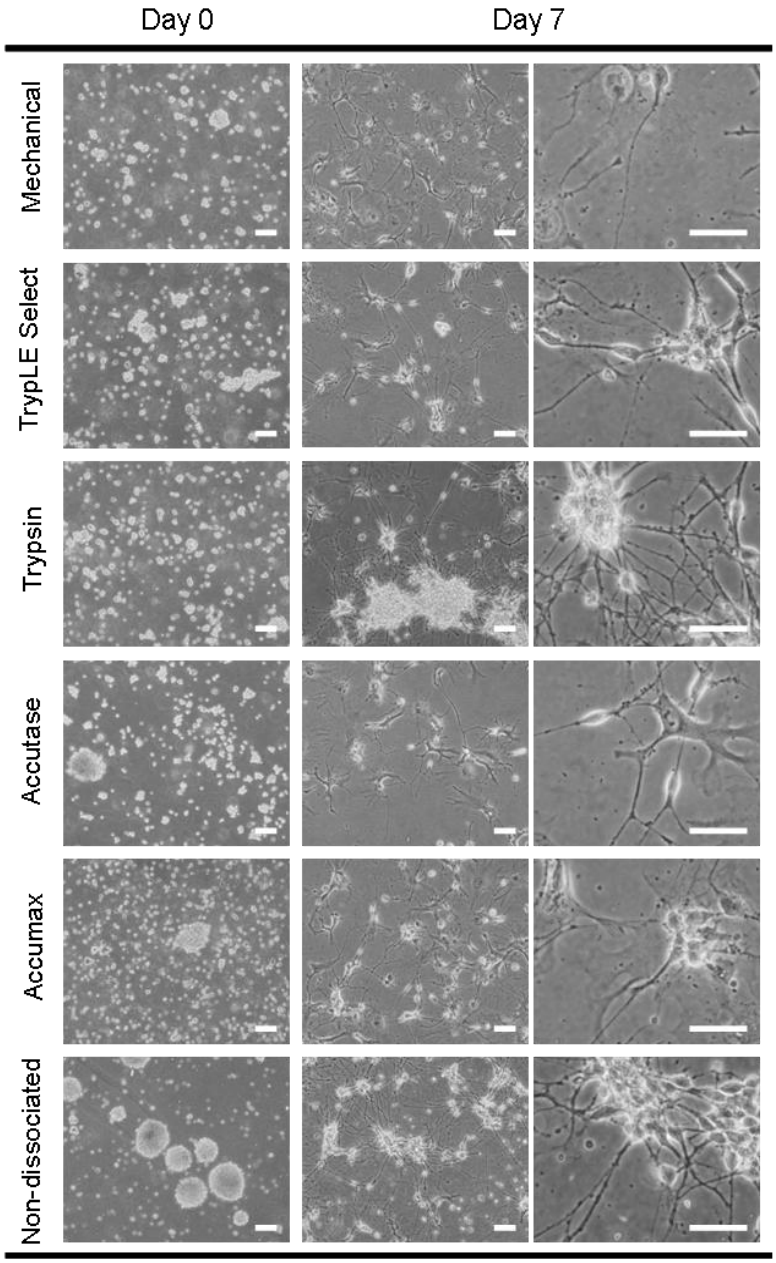

Figure 3.3. Phase contrast micrographs showing the neurosphere dissociated cultures obtained immediately after each dissociation procedure and following 7 days of culture under mitotic inhibitory conditions. The column in the right corresponds to a 4-fold magnification of the corresponding culture shown in the centre column. Scale bar: 50μm.

After 7 days of culture, it was observed that Trypsin dissociated cultures assured better NT2-N neuron preservation (Figure 3.3) as they yielded higher fractions of NT2-N neurons (quantified by cell counting after selective trypsinization, see Materials and Methods) from the total number of NT2 cells at neurosphere harvesting. This percentage of neuronal differentiation was higher on Trypsin dissociated cultures (approximately 6.8%) than on the others dissociated cultures (less than 1%), where smaller amounts of NT2-N neurons were detected on PDL-MG coated surfaces (Figure 3.3). Therefore, Trypsin digestion was selected as the best method for neurosphere dissociation since it promoted higher recovery of NT2-N neurons.

On the second strategy, non-dissociated neurospheres were directly plated into PDL-MG coated surfaces. After 7 days of culture, no cell aggregates were detected in the cultures plates. Besides that, higher amounts of NT2-N neurons with extensive neuritic networks were covering the entire surface. Here, the fraction of NT2-N cells achieved from the initial neurosphere harvesting was 12.3%, approximately 2 times higher than those reached in the trypsin dissociated cultures. These results suggested that harvesting non-dissociated neurospheres is the best strategy to recover NT2-N neurons.

Neurospheres and trypsin dissociated NT2 cells were also plated directly on uncoated tissue cultures flasks at harvesting time. However neither neurospheres nor dissociated cells attached to those untreated surfaces (results not shown).

3.3. NT2-N selection and characterization

Following neurospheres harvesting to coated surfaces, non-dissociated neurospheres cultures were further characterized in order to describe the changes and evolution of the cultures.

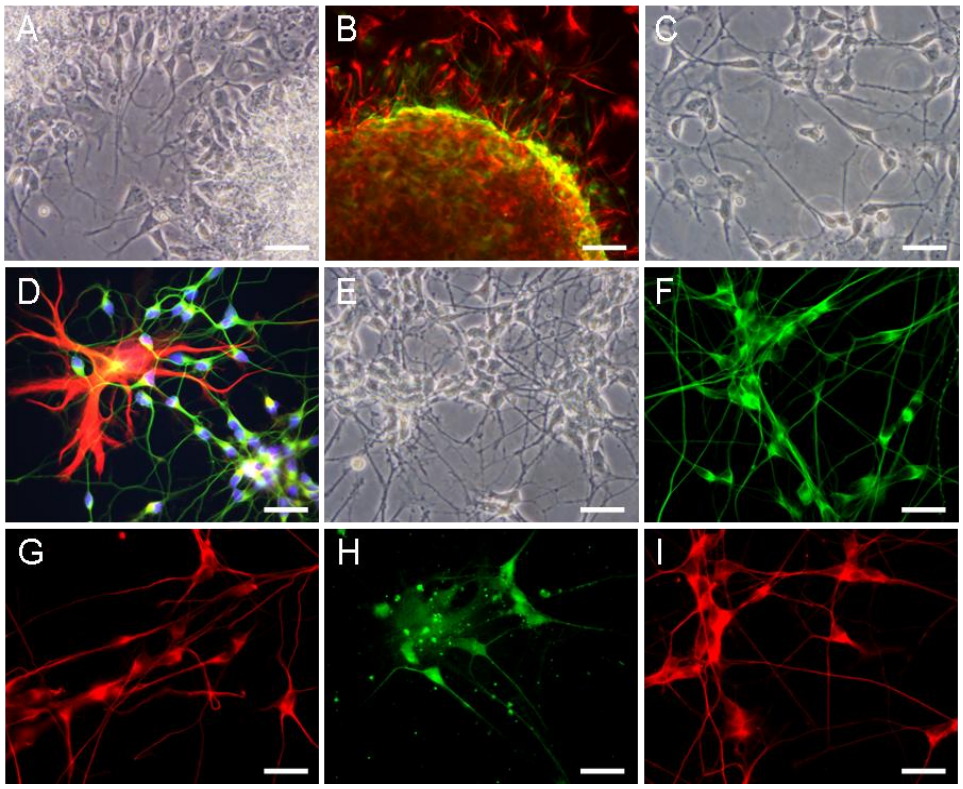

Figure 3.4. Characterization of NT2-N cultures. (A) Non-dissociated NT2 neurospheres adhered well on PDL-MG coated surfaces after 1 day of neurosphere platting. At this time, neurospheres sprout large numbers of non neuronal cells and also some neurons. (B) Immunofluorescence image of NT2 cultures after 1 day of neurosphere harvesting: Double labelling with β-TubIII (green) and nestin (red). (C) After 7 days, the culture became enriched into well differentiated NT2-N neurons. (D) Immunofluorescence images of NT2 cultures after 7 days of neurosphere harvesting: Double labelling with b-TubIII (green) and nestin (red). Nuclei are labelled with DAPI (blue). (E) Pure cultures of NT2-N neurons obtained upon selection. (F-I) Immunofluorescence images in pure NT2-N cultures: NT2-N neurons labelled with (F) β–TubIII, (G) NF-L, (H) MAP2a&b and (I) NF200. Scale bar: 50μm.

After 1 day of neurosphere platting, non-dissociated NT2 neurospheres adhered well on PDL-MG coated surfaces. During the first days, neurospheres sprout large numbers of non neuronal cells and also some NT2-N neurons; at this time, cultures are composed by stem cells, neural progenitor cells and differentiated cells as detected by phase-contrast

microscopy (Figure 3.4A). This observation was confirmed by double staining the cell cultures using antibodies specific for either the undifferentiated and neural progenitor NT2 cells (rabbit anti-nestin) or NT2-N neurons (mouse anti-β–TubIII) (Figure 3.4B). Incubation with mitotic inhibitory conditions led to an elimination of proliferating $NT2^-$ cells and the cultures gradually became enriched into differentiated NT2-N neurons and extensive neuritic networks. After 7 days of treatment, it was detected that 95% of the cells present in culture plates were NT2-N neurons; the rest of the cell population appeared as flat cells with extensive cytoplasms (Figure 3.4C,D). At this point, no cell aggregates were detected in the culture plates.

To further enrich for neurons, these cells were selectively dislodged (see Material and Methods) and plated into new culture dishes coated with PDL and MG. As shown in Figure 3.4E, pure cultures of NT2-N neurons with extensive neuritic networks covering the entire surface were obtained upon selection.

The identity of those final pure cultures was confirmed by positive staining for the neuronal markers NF-L, NF200, β-TubIII and MAP2a&b (Figure 3.4E-I). By the end of the culturing strategy, the cells were also stained for neural precursor cell (Nestin) and glial markers (GFAP for astrocytes and O4 for oligodendrocytes). Neither Nestin nor GFAP and O4 positive cells were detected in the final cultures of NT2-N cells (not shown).

3.4. Expansion and differentiation apparent rates of NT2 cells in stirred and static cultures

To assess the level of functionality of this novel NT2 differentiation process, the apparent rate of expansion (i.e. the rate of cell expansion obtained after RA treatment period – 35 days in static and 16 days in stirred culture) and the apparent rate of differentiation (i.e. the rate of cell differentiation

achieved during the RA treatment period- 35 days in static and 14 in stirred) were compared for the static and stirred suspension culture. For this, it was assumed that no NT2-N cells were present at inoculation day (day 0) and that differentiation occurs during incubation with RA. Subsequent steps for neurons selection and recover were not considered at the apparent differentiation rates calculation.

Figure 3.5 shows that the lengthy static adherent culture protocol promotes higher apparent rate of expansion (5.6×10^3 cell.mL^{-1}.h^{-1}) compared to cells cultured in stirred suspension vessels (2.5×10^3 cell.mL^{-1}.h^{-1}). Concerning the apparent ratio of differentiation the highest value was achieved in stirred suspension culture (5.4×10^2 NT2-N cell.mL^{-1}.h^{-1} comparing to 1.9×10^2 NT2-N cell.mL^{-1}.h^{-1} obtained in static culture). These findings suggest that higher amounts of contaminant cells are produced in static cultures.

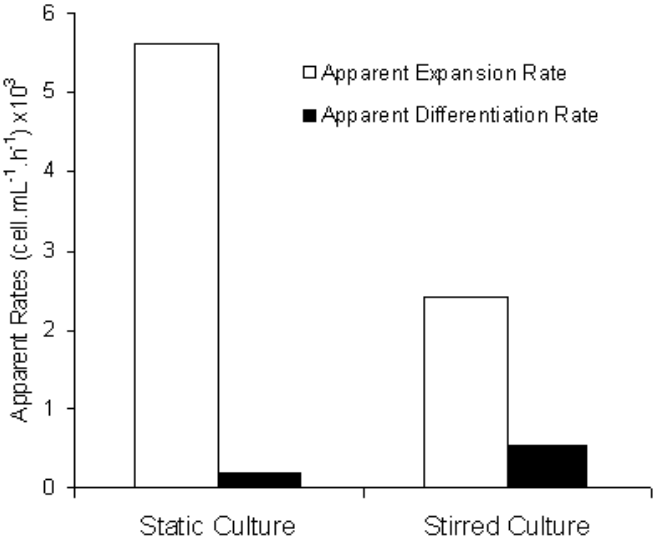

Figure 3.5. Apparent rates of NT2 cell expansion (white bars) and neuronal differentiation (grey bars) obtained by static and stirred (spinner culture) differentiation protocols.

Due to the specific limitations of each culture system (surface area in static system and minimal cell concentration in stirred culture) different cell inoculation densities were used, approx. 2×10^5 cell/mL and 5×10^5 cell/mL for static and stirred culture, respectively. Therefore the apparent differentiation rates results suggest that the neuronal differentiation potential of both culture strategies was practically similar.

3.5. Neuronal differentiation of NT2 cells in stirred and static cultures

At the end of each neuronal differentiation strategy, the final concentrations of NT2-N neurons were calculated. Table 3.2 shows that higher concentrations of NT2-N neurons were achieved by the stirred culture strategy in 23-28 days (1.8×10^5 cells/mL). In the lengthy static culture protocol the final concentration of NT2-N neurons was about 1.6×10^5 cell/mL, after 47-53 days of culture.

Table 3.2. Comparison of static and stirred culture conditions for NT2 neuronal differentiation.

	Static Culture	**Stirred Culture**
Differentiation Time	47 days	23 days
NT2-N final concentration	1.6×10^5 cell/mL	1.8×10^5 cell/mL
Differentiation Efficiency	3.2 ± 0.8 %	13.6 ± 1.8 %
Laborious	Yes	No
Scalable	No	Yes

Neuronal differentiation efficiency was also compared by analyzing the ratio between the number of reached NT2-N neurons and the total amount of NT2 cells harvested after RA treatment period (Table 3.2). For this, three experimental trials were evaluated for each differentiation

system.Reproducible results showed that the lowest value of differentiation efficiency was achieved by the static culture protocol (3.2 ± 0.8 %) which was shown to be about four times less efficient than the stirred culture protocol (13.6 ± 1.8%).

4. DISCUSSION

Several static culture protocols used for NT2 differentiation have been proposed to date. However, they were found to be time consuming, extremely laborious, to produce low yields of neurons per culture and not to be scalable (Pleasure et al., 1992; Durand et al., 2003; Horroks et al., 2003). Herein, a novel strategy to expand, accelerate and enhance the neuronal differentiation process of NT2 cells was developed, overcoming those process limitations. Therefore, the challenge was to improve the proliferation and differentiation steps, by culturing NT2 cells as 3D aggregates in the presence of RA, using suspension stirred bioreactors, without compromising their differentiation potential.

Culturing NT2 cells in spinner flasks yielded neurospheres with homogenous morphology (Figure 3.1) and avoided neurosphere clumping, resulting in a more efficient NT2 population than found in static cultures (Pleasure et al., 1992; Durand et al., 2003).

Moreover, the protocol developed drastically improves neuronal differentiation efficiency as compared with static cultures; a 4-fold increase of NT2 cells expressing several neuronal markers together with a reduced time required for the differentiation process is achieved (Table 3.2, Figure 3.5). The finding that NT2 differentiation is faster and more efficient in suspension than in adherent systems agrees with the results previously reported by Durand et al (2003). Although the studies reported by Durand et al. were performed in static conditions the authors clearly show that both

cell-cell contacts (within the neurospheres structures) and RA contribute to a more rapid neuronal differentiation process than in the conventional protocol using cell monolayer culture (Pleasure et al., 1992).

The decrease in the NT2 apparent expansion rate obtained for the suspension stirred system does not compromise the differentiation potential of NT2 precursors, suggesting that the proposed culturing protocol directs more efficiently the neuronal differentiation process reducing the percentage of contaminant cells (Figure 3.5). These are important achievements towards the development of scaleable protocols for stem cell differentiation; i.e. significant improvement of differentiation process efficiency and, ultimately reaching higher concentrations of Human Neurons, one of the current drawbacks of stem cell technology (revised in Kallos et al., 2003 and Ulloa-Montoya et al., 2005).

After NT2 expansion and differentiation, an efficient neurosphere harvesting process is required to assure a good recovery of differentiated neurons. Several protocols for NT2 cells and neurosphere dissociation have been proposed to date (Pleasure et al., 1992; Sen et al, 2004). However, due to the sensibility of neuronal cells and neurosphere complexity, the preservation of NT2 neurons after each dissociation procedure could be compromised. In the present study, both mechanical and enzymatic methods were evaluated, trypsin digestion being selected as the best strategy for neurosphere dissociation and NT2-N preservation. Nevertheless, the highest recovery of NT2-N neurons was achieved when non-dissociated neurospheres were collected directly to treated surfaces (with PDL-MG). This result indicates that both neurosphere dynamics and the matrix surfaces (PDL-MG) are sufficient to provide an optimal system for NT2-N neuron selection in a second step to optimize the neuronal differentiation process.

Overall, this study demonstrates that culturing human NT2 cells in stirred tank bioreactors increase by 4-fold culture differentiation efficiency. Furthermore, the possibility to include an additional period for precursor cells proliferation is also of great advantage, as it brings flexibility in what concerns the possibility to expand precursor NT2 cells before induction of neuronal differentiation, yielding higher numbers of final NT2-N cells per volume of culture. Static systems do not allow this design as culture surface limits the NT2 neuronal differentiation potential.

In conclusion, this culture method is promising for NT2 proliferation and differentiation, assuring a robust, easy to operate, scalable and reproducible system to culture NT2 pluripotent cells under a fully controlled environment.

Studies on bioprocess optimization (including the effect of several process parameters such as pO_2, medium composition, growth factor concentrations, feeding strategies, agitation rate) are ongoing aiming for further improvement of NT2 differentiation yields. Hopefully, the knowledge gained using this cell model will allow for a more straightforward application for the culture of human ES cells in bioreactors, a challenge of stem cells bioengineering.

5. ACKNOWLEDGEMENTS

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CHAPTER 4

INTEGRATING STEM CELL EXPANSION AND NEURONAL DIFFERENTIATION

This chapter was based on the following manuscript:

Serra, M., Brito, C., Costa, E.M., Sousa, M.F. and Alves, P.M., 2009. Integrating human stem cell expansion and neuronal differentiation in bioreactors. BMC Biotechnol. 9, 82.

ABSTRACT

Human stem cells are cellular resources with outstanding potential for cell therapy. However, for the fulfillment of this application, major challenges remain to be met. Of paramount importance is the development of robust systems for *in vitro* stem cell expansion and differentiation. In this work, we successfully developed an efficient scalable bioprocess for the fast production of human neurons.

The expansion of undifferentiated human embryonal carcinoma stem cells (NTera2/cl.D1 cell line) as 3D-aggregates was firstly optimized in spinner vessel. The media exchange operation mode with an inoculum concentration of $4x10^5$ cell/mL was the most efficient strategy tested, with a 4.6-fold increase in cell concentration achieved in 5 days. These results were validated in a bioreactor where similar profile and metabolic performance were obtained. Furthermore, characterization of the expanded population by immunofluorescence microscopy and flow cytometry showed that NT2 cells maintained their stem cell characteristics along the bioreactor culture time.

Finally, the neuronal differentiation step was integrated in the bioreactor process, by addition of retinoic acid when cells were in the middle of the exponential phase. Neurosphere composition was monitored and neuronal differentiation efficiency evaluated along the culture time. The results show that, for bioreactor cultures, we were able to increase significantly the neuronal differentiation efficiency by 10-fold while reducing drastically, by 30%, the time required for the differentiation process.

The culture systems developed herein are robust and represent one-step-forward towards the development of integrated bioprocesses, bridging stem cell expansion and differentiation in fully controlled bioreactors.

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1. INTRODUCTION

Many neurodegenerative disorders, such as Parkinson's disease, are caused by the impairment or death of neurons in the central nervous system (Storch and Schawarz, 2002). In the future, it is hoped that large numbers of stem cell-derived neurons will be produced in culture with the purpose of being used in clinical applications (Jones and Thomson, 2000). Hampering the faster implementation of the ambitious stem cell therapy technology, there is still the need of efficient, robust and scalable bioprocesses for cell expansion and/or differentiation *in vitro*.

During the last five years, substantial progress has been made towards this goal (King and Miller, 2007; Ulloa-Montoya et al 2005). Stirred suspension systems have been pioneered, by others and ourselves, as a promising *in vitro* system for stem cell expansion (Serra et al., 2009; Cormier et al., 2006), embryoid body cultivation (Niebruegge et al., 2009; Cameron et al., 2006) and stem cell differentiation into specific cell types (Serra et al., 2007). These systems offer attractive advantages of scalability and relative simplicity; stirring provides a more homogenous culture environment and allows the measurement and control of extrinsic factors such as nutrient and cytokine concentration, pH and dissolved oxygen (pO_2) (Zandstra and Nagy, 2001).

Aiming to improve the yields of specific stem cell stages, several culture parameters have been optimized, including the agitation rate, cell inoculum concentration and medium composition (King and Miller, 2007; Ulloa-Montoya et al., 2005; Zhao and Ma, 2005), and different culturing approaches have been developed such as the use of microcarrier supports (Serra et al., 2009) and cell encapsulation (Zhao and Ma, 2005). Perfusion and frequent feeding operation modes have been shown to increase the expansion of mesenchymal stem cells (Zhao and Ma, 2005), embryonic stem cells (Fong et al., 2005; Come et al 2008) and mammary epithelial

stem cells (Youn et al., 2006), without compromising their stem cell performance.

Computer-controlled bioreactors are particular advantageous for process development by allowing the online monitoring and control of specific culture parameters (temperature, pH and pO_2), ensuring a fully controlled environment for stem cell cultivation. Oxygen-controlled bioreactors have been used for culture of mouse and human ESC-derived cardiomyocytes (Niebruegge et al., 2009; Bauwens et a., 2005). Gilbertson *et al* (Gilbertson et al., 2006) were the first group to use controlled conditions for neural precursor cell culture as aggregates; the authors report the successful expansion of mouse neural stem cells in 500 mL bioreactors (temperature, pH and pO_2 control) while retaining the cell multilineage potential (Gilbertson et al., 2006). More recently, this system was applied to the culture of human neural precursor cells (Baghbaderani et al., 2008). The expansion of various human stem cell types in bioreactors under defined and controlled conditions remains to be addressed. Future challenges also include the combination of expansion and directed differentiation steps in an integrated bioprocess that will ultimately result in scale-up of well differentiated cells to clinically relevant numbers.

Within this context, the present work focused the development of a reproducible scalable system for the production of human neurons derived from expanded and differentiated stem cells. The human embryonal carcinoma cell line NTera-2/cl.D1 (NT2) was the cellular system used because it is a valuable model for both undifferentiated human embryonic stem cells (hESCs) (Andrews, 2002) and human neuronal differentiation *in vitro* (Przyborski et al., 2004). In addition, the neurons derived from this cell line have been successfully used in transplantation studies in several mouse models and in human stroke patients (Kondziolka and Wechsler,

2008), providing also promising material for cell therapy investigations in central nervous system.

Herein, undifferentiated NT2 cells were cultivated as 3D-aggregates in controlled stirred suspension conditions. In order to improve the yields of stem cells, two parameters were studied: (i) the inoculum concentration, as it has been shown to be critical in enhancing cell aggregation and culture profile (Cormier et al., 2006), and (ii) the culture operation mode, since it has been demonstrated that the feeding strategy affects cell metabolism and consequently could improve cell culture performance (Zhao and Ma, 2005; Bauwens et al., 2005; Xie and Wang, 2006). At the end, the expansion of undifferentiated NT2 cells, followed by directed neuronal differentiation were integrated in stirred bioreactors with temperature, pH and pO_2 control, in an effort to develop a promising model system for the production of human stem cell derivatives.

2. MATERIAL AND METHODS

2.1. Cell culture

NTERA-2/cl.D1 cells (NT2) were obtained from the CNDR, University of Pennsylvania School of Medicine. Undifferentiated NT2 cells were routinely cultivated in standard tissue culture flasks (Nunc) and maintained in OptiMEM medium (Invitrogen) supplemented with 5% (v/v) of fetal bovine serum (FBS, Hyclone) and 100 U/mL of penicillin- streptomycin (P/S, Invitrogen), according to method described at Brito *et al.*(Brito el al., 2007).

2.2. Stirred Suspension Culture

2.2.1. Undifferentiated NT2 cell expansion in spinner vessels

Undifferentiated NT2 cells (passage 60-62) were cultured as 3D-aggregates in 125-mL spinner vessels (Wheaton) equipped with a ball impeller and maintained at 37ºC and 5% CO_2 for up to 7 days. The agitation rate was increased during cultivation in order to avoid aggregate clumping and to control aggregate size (day 0 to 2 – 60 rpm, day 2 to 3 – 70 rpm, day 3 to 4 – 80 rpm, day 4 upwards – 90 rpm). Two independent experiments were performed for each expansion strategy.

Inoculum Concentration Experiments – Cells were cultured in a batch operation mode in Dulbecco's Modified Eagle's Medium- High Glucose (DMEM-HG, 25 mM glucose) (Invitrogen) supplemented with 10% (v/v) FBS and 100 U/mL of P/S (complete DMEM-HG). The cell inoculum concentrations evaluated were: $0.4x10^5$, $1x10^5$ and $4x10^5$ cell/mL; for an easier reading the nomenclature used was SP-0.4B, SP-1B and SP-4B, respectively. In SP-0.4B and SP-1B, cells were cultured in 75 mL of medium at 50 rpm during the first 4-8 h, to promote cell aggregation.

Culture Operation Mode Experiments – Glucose fed-batch and medium exchange culture operation modes were performed using an inoculum cell density of $4x10^5$ cell/mL; the nomenclature used for these experiments were SP-4FB (SP- spinner, FB- fed-batch) and SP-4ME (SP- spinner, ME-media exchange), respectively. In SP-4FB, the culture medium was DMEM-Base (Sigma) supplemented with 10% (v/v) FBS, 4mM of glutamine (Invitrogen), 100 U/mL P/S and 1.4 mM of glucose (Merck). During culture time, glucose concentration was monitored twice a day and maintained at lower levels (<1.4 mM); refeeds were performed accordingly to the consumption rates (calculated from 2 consecutive samples). SP-4ME was cultured in similar conditions to those described for SP-4B, except that

medium was partially exchanged daily from the day 3 onwards as follows: fifty percent of culture media was collected in sterile conditions and centrifuged at 200x *g* for 5 min; the supernatant was discarded and the recovered cell aggregates gently resuspended in an equivalent volume of pre-warmed complete DMEM-HG.

For all spinner cultures, sampling (2.5 mL) was performed 4 h after inoculation and daily from then on. Cell aggregates were monitored under an inverted microscope (Leica DM IRB). Cell concentration, metabolite concentration and lactate dehydrogenase activity were analyzed as described below.

2.2.2. NT2 culture in a fully controlled bioreactor

To ensure fully controlled cell culture environment, a stirred tank bioreactor (Santos et al., 2005) equipped with ball impeller and pH and dissolved oxygen (pO_2) measuring probes (Mettler-Toledo) was used for the expansion and differentiation of NT2 cells. The pH was kept at 7.2 by injection of CO_2 and addition of base (NaOH, 0.2 M). The pO_2 was maintained at 25% via surface aeration. The temperature was kept at 37ºC by water recirculation in the vessel jacket controlled by a thermocirculator module. Data acquisition and process control were performed using MFCS/Win Supervisory Control and Data Acquisition (SCADA) software (Sartorius-Stedim, Germany).

NT2 cell expansion – The SP-4ME experiment was reproduced in the bioreactor system, using undifferentiated NT2 cells with 60-62 passages in static conditions. Moreover, cells used for the inoculum (day 0) and at days 3 and 6 of cultivation in the bioreactor, were characterized using immunofluorescence tools and the neuronal differentiation potential evaluated (see below).

NT2 neuronal differentiation – Undifferentiated NT2 cells with up to 62 passages in static conditions were expanded in the bioreactor, in complete DMEM-HG, using an inoculum concentration of $4x10^5$ cell/mL. Differentiation was initiated in the middle of the exponential phase (day 3), following the differentiation protocol developed by Serra et al (Serra et al., 2007). Briefly, neuronal differentiation was induced by addition of retinoic acid (RA, Sigma) to the culture media, at a final concentration of 10 µM. A 50% media exchange was performed 3 times a week on a regular basis for up to 24 days. Two bioreactor independent experiments were performed.

Samples were collected from the bioreactor at 3 time points: day 9, 16 and 23 (corresponding to 1, 2 and 3 weeks of differentiation process). Cell concentration and neurosphere size were determined and culture was characterized using immunofluorescence microscopy. Neurospheres harvested at the referred time points were transferred to coverslips or culture flasks ($5x10^4$ cell/cm^2) coated with poly-D-lysine (PDL, Sigma) and Matrigel (MG, Becton-Dickinson) and cultured for up to 7 days in mitosis inhibitor (MI) medium: DMEM-HG supplemented with 5% FBS, 100 U/mL of P/S, 1 µM cytosine arabinosine (Sigma), 10 µM fluorodeoxyuridine (Sigma) and 10 µM uridine (Sigma). Neurons were selectively trypsinized (22,23) using a 0.015% Trypsin-EDTA solution (prepared from Trypsin-EDTA 1X, liquid 0.05% Trypsin, Invitrogen), counted and transferred to coverslips coated with PDL and MG for characterization by immunocytochemistry. Neuronal differentiation efficiency was defined as the ratio between the number of neurons obtained after 7 days of culture in MI medium and the total amount of cells harvested at the 3 different harvesting times.

Figure 4.1 summarizes the experimental outline used for expansion and differentiation processes.

2.3. Analytical methods

2.3.1. Cell concentration determination

Cell aggregates were dissociated by a 2 min incubation with Trypsin-EDTA (0.05%) at 37ºC followed by cell resuspension in complete DMEM-HG. Cell density was assessed using a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany) and cell viability estimated by the standard trypan blue exclusion test.

2.3.2. Aggregate diameter

Aggregate size in each culture sample was determined using a micrometer coupled to an inverted microscope (Leica, DM IRB). Two perpendicular diameters of a minimum of 15 aggregates were measured and the average diameter was calculated. Aggregates less than 20 μm in diameter (generally cell doublets or triplets) were not considered for calculations as they represent a small percentage of the total cell number in culture.

2.3.3. Lactate dehdrogenase activity

Lactate dehydrogenase (LDH) activity from the culture supernatant was determined as an indirect way of assessing cell death. LDH activity was determined by following spectrophotometrically (at 340 nm) the rate of oxidation of NADH to NAD^+ coupled with the reduction of pyruvate to lactate.The specific rate of LDH release (q_{LDH}, $U.day^{-1}.cell^{-1}$) was calculated for every time interval using the following equation: $q_{LDH} = \Delta LDH/(\Delta t\, \Delta X_V)$, where ΔLDH (U) is the change in LDH activity over the time period Δt (day) and ΔXv (cell) is the average of total cells during the same time period.

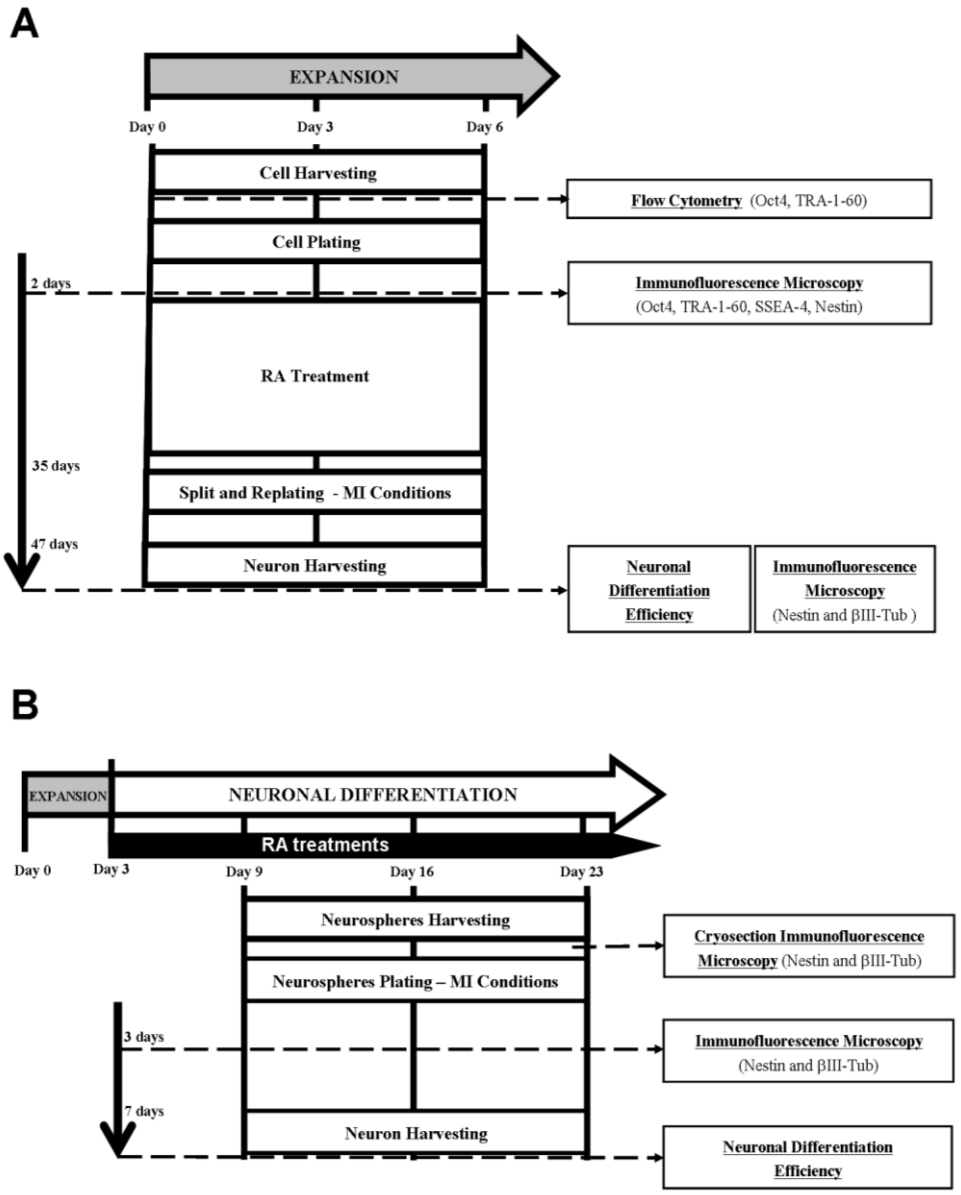

Figure 4.1. Experimental outline for NT2 cell sampling and characterization during expansion (A) and differentiation (B) in fully controlled bioreactors. (A) In expansion runs, cells were harvested from days 0 (inoculum), 3 and 6 and immediately characterized by flow cytometry. Harvested cells were plated on glass coverslips and processed for immunofluorescence microscopy analysis after 2 days or plated in tissue culture flasks for induction of neuronal differentiation. For this, cultures were treated with retinoic acid (RA) for 5 weeks, splitted and further cultured in mitosis inhibitory (MI) conditions. After 12 days in MI, the neurons were harvested,

identified by immunofluorescence microscopy using neuronal markers and neuronal differentiation efficiencies were calculated. (B) In differentiation runs, the addition of RA was initiated at day 3 of bioreactor culture and prolonged for 3 weeks. Neurospheres were harvested at day 9, 16 and 23. The latest were analyzed by cryosection immunofluorescence microscopy. All neurosphere harvested were plated in static culture flasks and cultured in MI conditions. After 3 days, cultures were characterized by immunofluorescence microscopy and after 7 days and neuronal differentiation efficiencies were calculated.

The cumulative value q_{LDHcum} was estimated by $q_{LDHcum\ i+1} = q_{LDH\ i} + q_{LDH\ i+1}$. The fold increase of the specific LDH release rates achieved throughout 6 days of cultivation were determined by calculating the ratio between the values of q_{LDHcum} obtained at day 6 and day 0. These values indirectly represent the fold increase in cell lysis obtained within 6 days of culture.

2.3.4. Metabolite analysis

Glucose (GLC), lactate (LAC) and glutamine (GLN) concentrations in the culture medium were analyzed using an YSI 7100MBS (YSI Incorporated, USA). Ammonia was quantified enzymatically using a commercially available UV test (Roche, Germany).

The specific metabolic rates ($q_{Met.}$, $mol.day^{-1}.cell^{-1}$) were calculated using the equation: $q_{Met.} = \Delta_{Met}/(\Delta t\ \Delta X_v)$, where Δ_{Met} (mol) is the variation in metabolite concentration during the time period Δt (day) and ΔX_v (cell) the average of adherent cells during the same time period.

2.3.5. Apparent growth rate and fold increase in cell expansion

Apparent growth rates and fold increase parameters were calculated for all expansion cultures. Apparent growth rates (μ, day^{-1}) were calculated using a first order kinetic model for cell expansion: $dX/dt = \mu X$, where t (day) is the culture time and X (cell) is the value of viable cells for a specific t. The μ values were estimated applying the model to the slope of the curves during

the exponential phase. The fold increase in cell expansion (FI) was defined as the ratio X_{MAX}/X_0, where X_{MAX} is the peak cell density (cell/mL) and X_0 is the inoculation cell density (cell/mL).

2.4. Differentiation potential

To assess the neuronal differentiation potential along the expansion assays, $2.3x10^6$ cells were collected from the suspension cultures and plated in a T75 flask (Nunc). NT2 cells were differentiated into post-mitotic neurons according to Pleasure et al (Pleasure et al., 1992). Briefly, cells were cultured for 5 weeks in complete DMEM-HG supplemented with 10 µM RA. Cells were splitted at 1:4.5 ratio and cultured in MI medium for 12 days. After this period, neurons were selectively trypsinized, as described above, counted and transferred to coverslips coated with PDL and MG for characterization by immunocytochemistry. Neuronal differentiation efficiency was defined as the ratio between the number of neurons obtained after culture in MI medium and the total amount of cells harvested after RA treatments.

2.5. Immunofluorescence microscopy

In expansion cultures, cell aggregates were collected at day 3 and 6, dissociated using Trypsin-EDTA (0.05%) at 37ºC followed by cell resuspension in complete DMEM-HG, and transferred to glass coverslips. Three days after plating, cultures were characterized. In differentiation assays neurospheres were harvested from the bioreactor cultures at day 9, 16 and 23, and processed for cryosection or transferred to coverslips coated with PDL and MG (see Figure 4.1).

Cells in coverslips were washed in PBS with 0.5 mM $MgCl_2$ and fixed in 4% (w/v) paraformaldehyde solution in PBS with 4% (w/v) sucrose, for 20 min. For cryosection, neurospheres were washed in PBS, transferred to a tissue

protecting compound (Tissue Teck, OCT™ Compound) and frozen at -80ºC. Ten µm sections, obtained using a cryostat (Leica), were rehydrated with PBS and fixed in methanol, at -20ºC, for 10 min. After fixation, the same procedure was followed for cryosections and coverslips.

For staining intracellular epitopes, cells were permeabilized with 0.1% (w/v) Triton X-100 (TX-100) in PBS, for 15 min. After 1 h in blocking solution (0.2% (w/v) fish skin gelatin in PBS), cells were incubated with primary antibody for 2 h. The coverslips were washed 3 times with PBS and overlaid with secondary antibody for 1 h. Primary and secondary antibodies were diluted in 0.125% (w/v) fish skin gelatin in PBS with 0.1% (w/v) TX-100. Samples were mounted in ProLong mounting medium (Molecular Probes), supplemented with DAPI for nucleus staining. For surface epitopes staining, cells were not permeabilized with TX-100. Samples were visualized using a fluorescence microscope (Leica DMRB).

Primary antibodies used were: mouse anti-tumor related antigen-1-60 (Tra-1-60) (Santa Cruz Biotechnology), mouse anti-stage specific embryonic antigen-4 (SSEA-4) (Santa Cruz Biotechnology), mouse anti-Oct-4 (Santa Cruz Biotechnology), mouse anti-nestin (Chemicon), mouse anti-type III β-tubulin (βIII-Tub) (Chemicon), mouse anti-microtubule associated protein 2A and 2B (MAP2) (Chemicon). The secondary antibodies were goat anti-mouse IgM-AlexaFluor488, goat anti-mouse IgG-AlexaFluor 594, goat anti-mouse IgG-AlexaFluor 488 and rabbit anti-mouse IgG-AlexaFluor 594 (Invitrogen).

2.6. Flow cytometry

Cells used for the inoculum (day 0) and from day 3 of the bioreactor expansion culture were dissociated into single cells and analyzed by flow cytometry. Samples were fixed in CytofixCytoperm reagent (BD Pharmigen) for 10 min, blocked with 1% BSA in PBS at 4ºC for 30 min and, in the case

of intracellular antigens, permeabilized with 1% TX-100 for 10 min. Primary antibodies were mouse anti-Tra-1-60 and anti-Oct-4. Secondary antibodies were anti-mouse IgM-AlexaFluor488 and anti-mouse IgG-AlexaFluor488. Ten thousand events were registered per sample with a CyFlow®space (Partec) instrument, using the appropriate scatter gates to avoid cellular debris and aggregates. A cell was considered to be positively stained if the measured fluorescence intensity exceeded the signal obtained by cells incubated with an isotype control antibody (Santa Cruz Biotechnology).

2.7. Statistical analysis

For each spinner and bioreactor assays, two independent experiments were performed. The results were expressed as the mean ± standard deviation. The statistical test used, One-way ANOVA, was performed in SPSS 13.0 for Windows for a level of confidence of 95% (a=0.05) followed by the Scheffé multiple comparison test.

3. RESULTS

With the goal of developing a robust and scalable system for NT2 neuronal differentiation, both expansion and differentiation steps were integrated in a fully controlled bioreactor process. Firstly, different strategies for expansion of undifferentiated NT2 cells as 3-D aggregates were screened in stirred spinner vessels; two parameters were studied (i) the inoculum concentration and (ii) the culture operation mode, i.e., medium replenishing strategies. Having the expansion of pluripotent NT2 cells optimized and well characterized, the neuronal differentiation strategy previously developed by our group (Serra et al., 2007), was integrated and the overall bioprocess combined in the bioreactor.

3.1. Effect of inoculum concentration in NT2 cell expansion

Three different cell inoculum concentrations were tested in batch culture mode, using 125 mL spinners: 0.4, 1 and 4 x10^5 cell/mL (SP-0.4B, SP-1B and SP-4B, respectively).

During the first 24 h of SP-1B and SP-4B cultures, cells assembled into small 3D-aggregates (Figure 4.2A) ranging from 40 to 65 µm. After this period, cells started to divide and aggregate size increased up to 150 µm. The growth curve and the calculated apparent growth rates are shown in Figure 4.2B and Table 4.1, respectively. SP-1B exhibited a high apparent growth rate (0.51 $\pm$ 0.01 day^{-1}) and the highest FI in cell concentration (7.14 $\pm$ 0.86). Nevertheless, maximum cell density 6.64 ($\pm$ 1.57) × 10^5 cell/mL was only reached 6 days after inoculation, whereas in SP-4B, a maximum of 8.48 ($\pm$ 0.11) × 10^5 cell/mL was achieved at day 3. From day 4 onwards of SP-4B culture, cells started to detach from the aggregates (Figure 4.2A), resulting in cell death (data not shown). Similar behavior was observed for SP-1B culture upon day 7 of cultivation. Concerning the SP-0.4 culture, cell aggregates were rare and small throughout cultivation time (Figure 4.2A). In fact, no effective cell growth was observed (Figure 4.2B) and cell viability was low (data not shown).

Aiming to develop an efficient bioprocess for the fast production of human neurons, cell number and culture time were the parameters preferentially used to select the best strategy. For SP-4B, the time needed to achieve X_{max} was 2 times lower than for SP-1B, reaching similar X_{max} values (Table 4.1). Based on these results, SP-4B was chosen to be further optimized and integrated with the neuronal differentiation step.

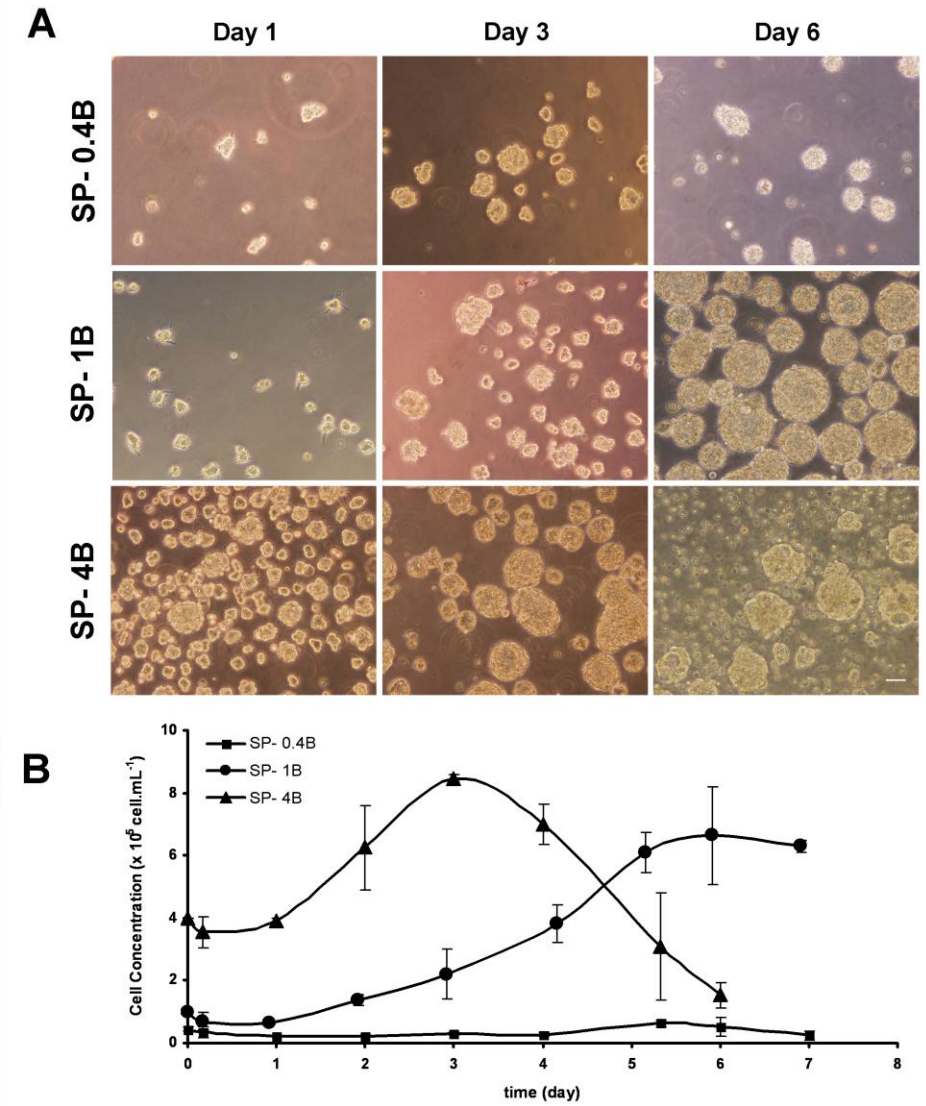

Figure 4.2. Effect of inoculum concentration in NT2 cell expansion as 3D-aggregates. Cells were cultured in spinner vessels with inoculum concentrations of 0.4 (SP-0.4B, squares), 1 (SP-1B, circles) and 4 (SP-4B, triangles) x10^5 cell/mL. Phase contrast photomicrographs of cultures samples visualized by day 1, day 3 and day 6 of cultivation. Scale bar: 100 µm (A). Growth curves expressed in terms of cell concentration; error bars denote standard deviation of average from 2 independent experiments (B).

Table 4.1. Growth kinetics of NT2 cell expansion as 3D-aggregates using different culture strategies. Apparent growth rate (μ), fold increase (FI) and maximum cell concentration values (X_{max})of NT2 cells cultured in spinner vessel (SP) or in bioreactor (BR); with inoculum densities of $0.4x10^5$ (SP-0.4) $1x10^5$ (SP-1) or $4x10^5$ cell/mL (SP-4, BR-4); in batch (B), fed-batch (FB) and media-exchange (ME) culture operation mode.

Strategy	μ (day^{-1})	FI	X_{max} ($\times10^5$ cell/mL)
SP-0.4B	n. a.	n. a.	0.63 ± 0.11 *
SP-1B	0.51 ± 0.01	7.14 ± 0.86 *	6.64 ± 1.57
SP-4B	0.39 ± 0.02	2.12 ± 0.03	8.48 ± 0.11
SP-4FB	0.52 ± 0.06	4.30 ± 0.33 *	17.19 ± 1.30 *
SP-4ME	0.41 ± 0.06	4.56 ± 0.04 *	18.25 ± 0.18 *
BR-4ME	0.37 ± 0.03	4.10 ± 0.41	16.25 ± 0.16

Apparent growth rate (μ) and fold increase (FI) values are expressed as mean ± SEM from n=2 independent experiments. n. a. – not applicable. *Indicates significant statistical difference (p-value < 0.05) from the SP-4B mean values of μ, FI and Xmax by the one-way ANOVA analysis with a Scheffé post-hoc multiple comparison test.

3.2. Impact of operation mode in NT2 cell expansion

In all batch cultures there was a rapid decrease in cell density after the culture reached its maximum concentration value (Figure 4.2A). Although no complete depletion of neither glucose nor glutamine was observed (Figure 4.3A,C), this profile could be correlated to the exhaustion of other essential nutrients and/or the progressive accumulation of toxic metabolic waste products such as lactate and ammonia (Figure 4.3B,D). In SP-4B, by the 4^{th} day of cultivation, the lactate and ammonia concentrations were already 21.9 mM and 3.1 mM, respectively (Figure 4.3B,D). In SP-1B, these values were also high at day 7 of culture (27.2 mM and 4.2 mM for lactate and ammonia concentration, respectively).

Aiming at prolonging the exponential growth phase and improve the cell expansion, two additional operation modes were tested. The first strategy consisted of a glucose fed-batch operation mode (SP-4FB). In this strategy, culture was initiated at low concentration of glucose (1.4 mM) and the

feeding was performed twice a day assuring the maintenance of low levels of glucose throughout cultivation time (see Methods section). The second strategy (SP-4ME) was designed to simulate a perfusion system, in which cells are kept in culture and the media is renovated regularly. This was achieved by performing a daily partial media exchange (50%) from the 3rd cultivation day onwards, as this time point corresponded to the growth peak in the batch culture (Figure 4.2B, SP-4B).

For SP-4ME and SP-4FB cultures, the exponential growth phase was extended until day 5 (Figure 4.3F), with a significant increase in X_{max}, when compared to SP-4B (Table 4.1). These differences are also reflected in cell metabolism, as shown by the nutrient consumption and metabolite production profiles (Figure 4.3E). The SP-4FB culture presented the lowest specific rates of glucose consumption and lactate production. The lower accumulation of lactate (16.5 mM at day 6, Figure 4.3B) in SP-4FB contributed to the high apparent growth rate of this strategy (0.52$\pm$0.06 day^{-1}, Table 4.1). Nevertheless, there was still a steeply decrease in cell concentration after day 6 (Figure 4.3F) that may result from the accumulation of other toxic metabolites, such as ammonia, which reached values as high as in SP-4B (4.0 mM and 4.2 mM for SP-4FB and SP-4B cultures, respectively, at day 6 of cultivation, Figure 4.3D).

Cell viability was calculated in term of cell lysis, translated by the specific release rates of the intracellular enzyme LDH (q_{LDH}). For SP4-ME, the q_{LDH} achieved were lower (fold increase of 9.1) than those obtained for SP-4B and SP-4FB (fold increase of 20.5 and 19.4, respectively) throughout 6 days of cultivation, indicating that a lower percentage of cell lysis occurred in the SP-4ME culture. Despite no complete depletion of either glucose or glutamine was observed in the strategies tested, cells in SP4-ME were not continuously subjected to the accumulation of toxic metabolites, which probably had a positive effect on cell viability (Figure 4.3A-D).

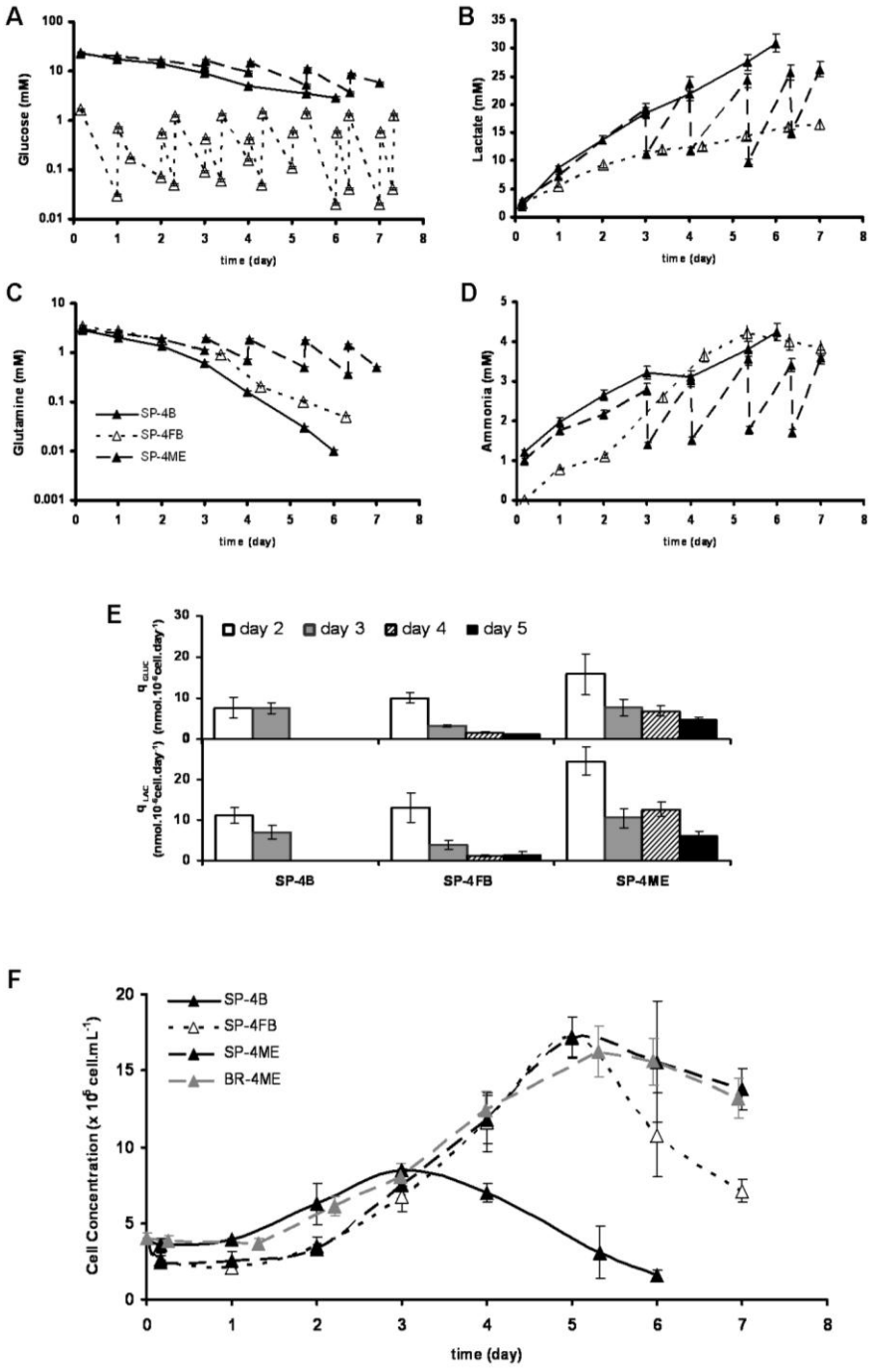
A
Glucose (mM)
time (day)
B
Lactate (mM)
time (day)
C
Glutamine (mM)
SP-4B
SP-4FB
SP-4ME
time (day)
D
Ammonia (mM)
time (day)
E
day 2
day 3
day 4
day 5
SP-4B
SP-4FB
SP-4ME
F
SP-4B
SP-4FB
SP-4ME
BR-4ME
Cell Concentration (x 10^6 cell.mL^-1)
time (day)

Figure 4.3. Effect of culture operation mode on NT2 cell expansion as 3D-aggregates. Cells were cultured in spinner vessels (SP) or in bioreactors (BR), with inoculum concentration of $4x10^5$ cell/mL, using different operation modes: batch (SP-4B, black line and triangles), fed-batch (SP-4FB, dashed line and white triangles) and media exchange (SP-4ME, dashed line and black triangles, and BR-4ME, grey line and triangles). Concentrations of glucose **(A)**, lactate **(B)**, glutamine **(C)** and ammonia **(D)** presented in media during culture time. Specific rates of glucose consumption and lactate production shown over the course of exponential growth phase **(E)** (day 2- white bars, day 3- grey bars, day 4-striped bars, day 5-black bars). Growth curves expressed in terms of cell concentration; error bars denote standard deviation of average from 2 independent experiments **(F)**.

3.3. Expansion and characterization of undifferentiated NT2 cells in a bioreactor

From the results shown above, SP-4ME was the most promising culture strategy for expansion of undifferentiated stem cell. The next step was the implementation of this strategy in a fully controlled 125 mL bioreactor, BR-4ME.

The growth curve obtained for the bioreactor run BR-4ME was comparable to the one obtained for the medium exchange operation mode in spinner SP-4ME; similar apparent growth rates and maximum concentrations were obtained (Figure 4.3F, Table 4.1). NT2 cells expanded in the bioreactor for 6 days were characterized in terms of pluripotency, undifferentiated phenotype and differentiation potential. The expression of stem cell markers (Oct-4, TRA-1-60, SSEA-4) and nestin, an intermediate filament protein associated with undifferentiated phenotype of NT2 cells (Pleasure and Lee, 1992), was detected during exponential growth phase (day 3) and at day 6 (Figure 4.4A). This labeling pattern was similar to the cell inoculum (day 0).

Moreover, in addition to the expression of stem markers analysis, the expanded cells ability to differentiate into neurons was also confirmed. For that purpose, cells were collected at 3 time points (day 0, 3 and 6) and

induced to differentiate into neurons using the standard static differentiation protocol (Pleasure et al., 1992). After treatment with RA and further cultivation in MI medium, the neuronal differentiation efficiency (defined as the ratio between the number of neurons obtained and the number of cells harvested from the bioreactor, see Methods section) was similar for all culture samples, presenting values in the range typically obtained for the static differentiation protocol (3.3±0.2%) (Serra et al., 2007). The differentiated neurons were identified by βIII-Tub and MAP2 positive staining (Figure 4.4B).

3.4. Integrating expansion and neuronal differentiation of NT2 cells in the bioreactor

Once the expansion of pluripotent NT2 cells was adapted and characterized in the bioreactor system, we further integrated the neuronal differentiation step according to Serra et al (Serra et al., 2007). Neuronal differentiation was induced by RA addition when cells achieved the middle of the exponential growth phase at day 3 (Figure 4.3C). Flow cytometry analysis of cell populations showed that the levels of Oct-4 (94.8% positive cells) and Tra-1-60 (88.7% positive cells) obtained for the inoculum were kept at day 3 of the bioreactor culture (97.2% and 94.6% Oct-4 and Tra-1-60 positive cells, respectively), confirming that the stem cell population was maintained at this time point.

Throughout differentiation, the aggregate size increased, reaching average diameters of 150 ± 40, 309 ± 94 and 458 ± 44 μm after 1, 2 and 3 weeks of RA treatment, respectively (Figure 4.5A,B,C, Table 4.2). The aggregate shape became uniform, forming compact and spherical structures (Figure 4.5B,C).

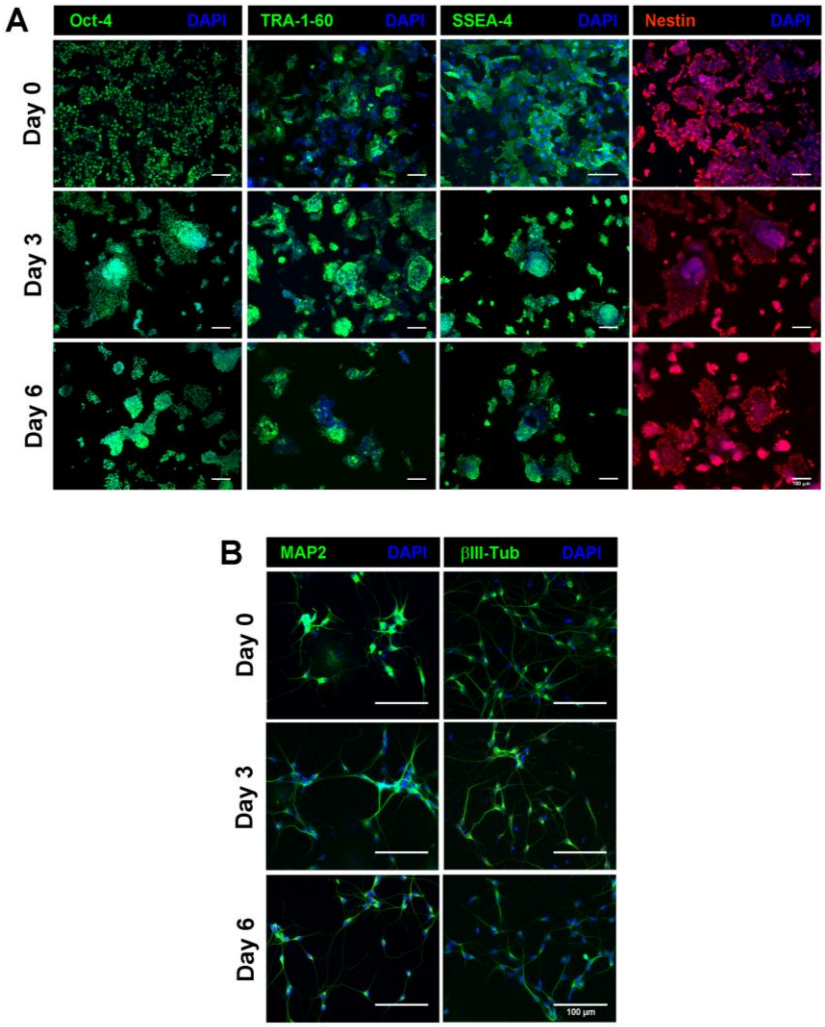

Figure 4.4. Characterization of NT2 cells expanded as 3D-aggregates. Immunofluorescence images of cells from the inoculum (day 0) and collected from the bioreactor culture (day 3 and day 6). Immunolabeling of Oct-4, TRA-1-60, SSEA-4 (green) and nestin (red). Nuclei are labeled with DAPI (blue) (**A**). Immunofluorescence images of differentiated cultures derived from the inoculum (day 0) and from the bioreactor culture (day 3 and day 6). Neurons labeled with βIII-Tub and MAP2 (green) (**B**). Nuclei were stained with DAPI (blue). Scale bars: 100 µm.

Table 4.2. Characterization of NT2 neurospheres cultured in a fully controlled bioreactor.

Neurospheres			
Time of harvesting (day)	9	16	23
Duration of RA treatment (week)	1	2	3
Neurosphere size (μm)	150 ± 40	309 ± 94	458 ± 44
Differentiation efficiency	0.13 ± 0.06	17.2 ± 2.2	37.4 ± 0.9

Neurosphere size and neuronal differentiation efficiency are expressed as mean ± SEM from n=2 independent bioreactor experiments

Immunofluorescence microscopy of aggregate cryosections showed that these were neurospheres, composed of precursors (nestin-positive) and differentiated neurons (βIII-Tub-positive), the latest distributed preferentially at the surface (Figure 4.5C1). After 9, 16 and 23 days of bioreactor culture (1, 2 and 3 weeks of neuronal differentiation, respectively), neurospheres were harvested and cultured for 7 days, on PDL-MG coated flasks, in MI medium, to allow cell migration and inhibit cell proliferation. One day post-seeding, the presence of neurites surrounding the neurospheres was more pronounced on cultures harvested at day 23 (Figure 4.5F), while on neurospheres harvested earlier, cells with flattened morphology predominated (Figure 4.5D). Three days post-seeding, the cell culture composition was analyzed by immunofluorescence microscopy (Figure 4.5G,H,I). Cultures derived from neurospheres harvested at day 23 were richer in neurons (βIII-Tub-positive staining) and presented more developed neuritic networks than the neurospheres harvested at day 16 (Figure 4.5H,I). A reduced number of βIII-Tub-positive cells was detected in cultures derived from neurospheres collected at day 9, in which nestin-positive cells predominated (Figure 4.5G).

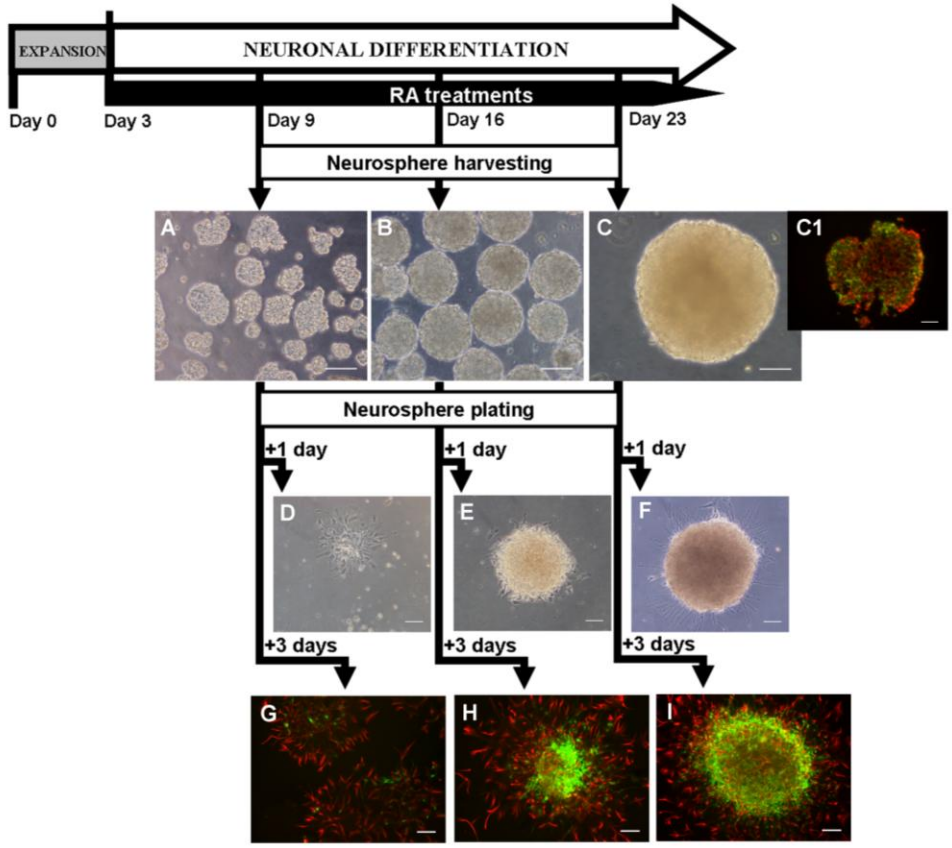

Figure 4.5. Neuronal differentiation of NT2 cells in a fully controlled bioreactor. Neuronal differentiation was induced by addition of retinoic acid (RA) from day 3 onwards (RA treatments). Phase contrast photomicrographs of neurospheres harvested at day 9 (**A**), day 16 (**B**) and day 23 (**C**) of the bioreactor culture. By day 23, neurosphere composition was analyzed by cryosection immunofluorescence microscopy - double labeling of nestin (red) and βIII-Tub (green) (**C1**). Harvested neurospheres were further cultured in mitotic inhibitory (MI) conditions, on poly-D-lysine and Matrigel-coated surfaces. Cultures were visualized by phase contrast microscopy 1 day after plating (**D**,**E**,**F**) and characterized by immunofluorescence microscopy 3 days after plating (**G**,**H**,**I**). Double labeling of nestin (red) and βIII-Tub (green). Phase contrast and immunofluorescence images of cultures derived from neurospheres harvested at day 9 (**D**,**G**), day 16 (**E**,**H**) and day 23 (**F**,**I**). Scale bars: 100μm**.**

The estimated neuronal differentiation efficiency was 0.13 ± 0.06 % and 17.2± 2.2% for cultures derived from neurospheres harvested at day 9 and 16, respectively (Table 4.2). The results obtained until day 16 were similar

to the ones described for the spinner culture (Serra et al., 2007), both in culture profile and differentiation efficiency, proving that the integrated culture strategy was successfully implemented in the bioreactor. Moreover, by extending the RA treatments for an additional week, a significant increase in the yield of neuronal differentiated cells was obtained (neuronal differentiation efficiency of 37.4 ± 0.9%, Table 4.2).

4. DISCUSSION

To fully fulfill the expectations raised by cell therapy it is urgent to develop robust and totally controlled culture systems, specially designed for the production of high numbers of differentiated and well characterized cells, expanded as fast and pure as possible. In the present study, we successfully developed a bioprocess for the rapid production of human neurons using fully controlled stirred tank bioreactors (125 mL). This was accomplished by integrating human NT2 cell expansion and differentiation in a two-step bioprocess.

In this particular study, an ideal expansion strategy should assure the fast production of high numbers of stem cells without compromising their potential. We demonstrated that, along expansion as 3-D aggregates, NT2 cells maintained their pluripotent and undifferentiated phenotype as well as the ability to differentiate into neurons. Different bioreaction parameters, including cell inoculum concentration and culture operation mode were studied. The results indicate $4x10^5$ cell/mL as the most adequate inoculum strategy to be integrated with the differentiation step, as it allowed higher cell densities in less culture time contributing to a fast overall process. However, the feasibility of starting the cultures with inoculum concentrations as lower as $1x10^5$ cell/mL looks promising for specific clinical applications in which the starting material is a limiting factor.

Although lower inoculation concentrations have been used to expand undifferentiated murine embryonic stem cells as aggregates (Cormier et al., 2006; zur Nieden et al., 2007), NT2 cell proliferation could not be achieved when 4×10^4 cell/mL were used. This difference in cell behavior may reflect the distinct cell origins, as NT2 are pluripotent human embryonal carcinoma stem cells, derived from teratocarcinomas (Pleasure et al., 1992), that closely resemble the human embryonic stem cells derived from the blastocyst inner cell mass (Henderson et al., 2002).

By using a fed-batch strategy, where low levels of glucose were maintained in culture, it was possible to enhanced glucose metabolism efficiency with a concomitant improvement of the FI in cell concentration and increase of culture lifespan. This strategy may have minimized the toxicity effect associated with lactate accumulation, as reported previously for several animal cell cultures (Xie and Wang, 1994; Cruz et al., 2000). Nevertheless, the accumulation of other toxic metabolites, including ammonia, resulted in an increase in cell death. The possible depletion of nutrients (others than glucose and glutamine) as well as the exhaustion of essential small molecules, namely growth factors, not replenished in the glucose fed-batch strategy, may have contributed to arrest cell growth. The media exchange mode overcame these drawbacks, being the most efficient strategy to enhance undifferentiated stem cell cultivation, as shown by the higher cell densities and higher culture viability obtained throughout the cultivation time. Therefore this strategy was chosen for implementation in the controlled bioreactor in which stem cell expansion was successfully reproduced, confirming the robustness of the process. Media exchange and perfusion strategies have been used previously for adult stem cell cultivation (King and Miller, 2007; Serra et al., 2007) and human embryoid bodies (Come et al., 2008). In order to achieve higher expansion ratios, as those obtained for the expansion process as aggregates of murine

embryonic stem cell (Cormier et al., 2006; zur Nieden et al., 2007) and human neuronal precursor cell (Burjghhghg et al., 2008), serial passage with addition of fresh media can be further included.

By incorporating both expansion and differentiation steps in an integrated bioprocess, this strategy also assures the feasibility of expanding human differentiated neurons derived from a continuous source of pluripotent stem cells. The system described herein allows for obtaining well differentiated neurons after 2 weeks of differentiation, as well as higher yields of neurons for a later culture time. Importantly, when compared to well established static differentiation protocols, this methodology drastically enhanced the neuronal differentiation efficiency of NT2 cells and reduced the time needed for differentiation process; for a differentiation time of 23 days in the bioreactor culture a 10-fold improvement in yield was observed over the static culture protocols lasting 35 days (Pleasure at al., 1992).

In this work, the expansion and differentiation of NT2 cells was successful validated in computer-controlled bioreactors. In future, further optimizations can be attempted aiming to determine the optimal conditions (pH, pO_2 and temperature) to grow and differentiate NT2 cells. So far, some studies have demonstrated that low pO_2 decreases the rate of stem cell differentiation and enhances stem cell proliferation (Gibbons et al., 2006). Nieruebuegge *et al.* also reported a significant increase in final cell number as well as an improvement of cardiac–enriched genes in hEBs cultures under hypoxic conditions (pO_2= 4%) (Nieruebuegge et al., 2009). A recent study reports that rat mesenchymal stem cell differentiation is enhanced at lower temperatures (32ºC) than in 37ºC conditions (Stolzing and Scutt, 2006).

5. CONCLUSION

In this work, a scalable and efficient two-step bioprocess for the generation of human NT2-derived neurons was developed in a fully controlled bioreactor, allowing continuous monitoring, non-invasive sampling and characterization. By integrating a fast expansion step with an efficient differentiation process, this strategy significantly reduced the time and improved the yields of the neuronal differentiation, when compared to the standard static differentiation protocols.

The controlled bioprocess developed herein can be adaptable to other cell types, including hESCs and iPSCs, representing a strong and promising starting point for the development of novel technologies for the production of differentiated derivatives from pluripotent cells.

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CHAPTER 5

IMPROVING EXPANSION OF PLURIPOTENT HUMAN EMBRYONIC STEM CELLS IN PERFUSED BIOREACTORS THROUGH OXYGEN CONTROL

This chapter was based on the following manuscript:

Serra, M., Brito, C., Sousa, M.F., Jensen, J., Tostões, R., Clemente, J., Strehl, R., Hyllner, J., Carrondo, M.J. and Alves, P.M., 2010. Improving expansion of pluripotent human embryonic stem cells in perfused bioreactors through oxygen control. J Biotechnol. 148(4), 208-15

ABSTRACT

The successful transfer of human embryonic stem cell (hESC) technology and cellular products into clinical and industrial applications needs to address issues of automation, standardization and the generation of relevant cell numbers of high quality. In this study, we combined microcarrier technology and controlled stirred tank bioreactors, to develop an efficient and scalable system for expansion of pluripotent hESCs.

We demonstrate the importance of controlling pO_2 at 30% air saturation to improve hESCs growth. This concentration allowed for a higher energetic cell metabolism, increased growth rate and maximum cell concentration in contrast to 5% pO_2 where a shift to anaerobic metabolism was observed, decreasing cell expansion threefold. Importantly, the incorporation of an automated perfusion system in the bioreactor enhanced culture performance and allowed the continuous addition of small molecules assuring higher cell concentrations for a longer time period. The expanded hESCs retained their undifferentiated phenotype and pluripotency.

Our results show, for the first time, that the use of controlled bioreactors is critical to ensure the production of high quality hESCs. When compared to the standard colony culture, our strategy improves the final yield of hESCs by 12-fold, providing a potential bioprocess to be transfered to clinical and industrial applications.

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1. INTRODUCTION

hESCs can provide a renewable source of cellular material for regenerative medicine, drug screening and *in vitro* toxicology (Davila et al., 2004; Jensen et al., 2009; Jones et al., 2000). However, the development of bioprocesses for production of hESCs or their progeny in large quantities will be necessary for their translation to these fields.

Expansion of undifferentiated hESCs typically requires adherence to a surface. Although the majority of culture methods available are still based on 2D surfaces (Ellerstrom et al., 2007), the use of microcarriers to support stem cell growth has been explored; recent studies showed the successful use of spinner vessels to expand hESCs on microcarriers, attaining higher cell concentrations than standard static cultures (Fernandes et al., 2009; Lock et al., 2009; Nie et al., 2009; Oh et al., 2009; Phillips et al., 2008; Kehoe et al., 2009). However, the impact of specific operating conditions upon expansion of pluripotent hESCs remains to be addressed. Oxygen is a critical factor in hESCs culture (Placzek et al., 2009) and there is emerging evidence suggesting that reducing oxygen concentration towards low levels (Fischer et al., 1993; Ottosen et al., 2006) is beneficial for the *in vitro* maintenance of pluripotent hESCs (Forsyth et al., 2006; Ezashi et al., 2005; Prasad et al., 2009). Additionally, perfusion mode is known to improve stem cell expansion assuring the renewal of nutrients and other factors and removal of metabolic byproducts (Bauwens et al., 2005; Serra et al., 2009a).

Herein, we demonstrated that microcarrier technology and controlled stirred bioreactors in perfusion mode, where scalability, automation, straightforward operation and tight control of the culture environment are combined, can be used to improve the expansion of pluripotent hESCs.

2. MATERIAL AND METHODS

2.1. Cell culture

hESCs cells (SCED^{TM}461, Cellartis AB) were routinely propagated in static conditions in standard culture medium (KO) (DMEM-KO supplemented with 20% (v/v) KOSR, 1% (v/v) MEM-NEAA, 0.1mM 2-mercaptoethanol, 2mM glutamax, 1% (v/v) Pen/Strep, 0.5% (v/v) gentamycin (all from Invitrogen) and 10ng/mL bFGF (Perprotech)) as previously reported (Ellerstrom, et al., 2007). For spinner and bioreactor cultures, hESCs were used at low passage numbers (6-8).

2.2. Spinner cultures

hESCs were inoculated at 1.5, 3 and 4.5 $\times 10^5$cell/mL into 125mL spinner vessels (Wheaton) containing Cytodex3^{TM} microcarriers (3g/L, GE Healthcare) coated with Matrigel^{TM} (BD Biosciences) [5,8]. Cells and beads were inoculated in 25mL of mouse embryonic fibroblasts conditioned medium (KO-CM) supplemented with 10µM Rock Inhibitor (Merck), and the spinner vessels were placed inside an incubator (37ºC, 5%CO_2) under intermittent stirring (vessels were agitated gently for 1 min every 30 min). After 6h fresh KO-CM was added to cultures and agitation rate set to 24rpm. By day 3, the volume was completed to 100mL; 50% of medium was replaced daily.

2.3. Bioreactor cultures

hESCs were cultivated in computer-controlled stirred tank bioreactors (BIOSTAT® Qplus, Germany), equipped with low shear stress marine 3-blade impellers, under defined conditions (working volume-300mL; pH-7.2; temperature-37ºC; pO_2-5% and 30% air saturation; surface aeration rate-0.1vvm; agitation rate-50-65rpm). Data acquisition and process control

were performed using Multiple Fermenter Control System for Windows Supervisory Control and Data Acquisition software (Sartorius Stedim, Germany). Cells (4.5×10^5 cell/mL) were seeded on microcarriers placed inside glass bottles and, after colonization (6-8h), transferred to bioreactor vessels. Semi-continous cultures were carried out by replacing 50% of the medium daily (1 pulse/day). Perfusion experiments were performed with a probe adapted to the bioreactor cap using automated gravimetric control (D= 0.5 day^{-1}, 30 pulses/day).

2.4. Cell growth and metabolism

Cell concentration, viability and microcarrier colonization were evaluated according to (Lock, et al., 2009). Lactate dehydrogenase activity, glucose and lactate concentrations in supernatants were measured as described before (Serra et al., 2009b). Growth kinetics and metabolic performance were determined according to (Serra, et al., 2009a). Briefly, apparent growth (μ) and death (k_d) rates were estimated using a simple first order kinetic model $dX/dt = \mu X$ and $dX/dt = k_d X$ respectively, where t (days) is the culture time and X (cells) is the value of viable cells for a specific t. μ and k_d were estimated using this model applied to the slope of the curves during the exponential and death phase, respectively. The specific metabolic rates ($q_{Met.}$, $mol.day^{-1}.cell^{-1}$) were calculated using the equation: $q_{Met.} = \Delta_{Met}/(\Delta t\, \Delta X_v)$, where Δ_{Met} (mol) is the variation in metabolite concentration during the time period Δt (day) and ΔX_v (cell) the average of viable cells during the same time period.

2.5. Immunocytochemistry

Immunocytochemistry was performed as described previously (Serra, et al., 2009b) and samples analyzed using a fluorescence microscope (DMI6000,

Leica). Primary antibodies used were: Tra-1-60, SSEA-4, Oct-4, Ki67, (all Santa Cruz Biotechnology), and hESCellect™ (Cellartis AB).

2.6. Flow cytometry

For flow cytometry analysis, cells were dissociated with TrypLE Select before labeling. Ten thousand events were registered per sample with a CyFlow®space (Partec) instrument as reported elsewhere [19]. Primary antibodies used were: Tra-1-60, SSEA-4, and isotype control antibodies (all Santa Cruz Biotechnology).

2.7. qRT-PCR

Total RNA was extracted from cells using RNeasy PLUS Mini kit (Qiagen) and cDNA synthesized with High Capacity cDNA synthesis kit (Applied Biosystems). PCR was performed using ABI7500 Real-Time PCR System, primers and probes from TaqMan Assays-on-Demand Gene Expression Products (Applied Biosystems).

2.8. Alkaline phosphatase analysis

Histological staining for alkaline phosphatase activity was carried out using a commercially available kit (Chemicon) following the manufacturer's instructions.

2.9. *In vitro* pluripotency test

hESCs were dissociated, transferred to non-adherent petri dishes (5×10^5cell/mL) and cultured in KO medium without bFGF. After 7 days, embryoid bodies (EBs) were transferred to gelatin coated plates and cultured for 14 days. Differentiated cells were identified using immunocytochemistry. Primary antibodies used were: α-smooth muscle

actin (α-SMA, DAKO), Forkheadbox A2 (FOX A2, Santa Cruz) and β tubulin type III (βIII-Tub, Chemicon).

2.10. *In vivo* pluripotency test

For teratoma formation, 1×10^6 cells expanded in bioreactors, were injected into the testis of 6-8 weeks old nude mice (6 mice used per sample). Eight weeks after injection the mice were sacrificed and the resulting teratomas examined histologically as described in (Oh et al., 2009).

3. RESULTS AND DISCUSSION

In this study we investigated the optimal conditions for propagation of pluripotent hESCs (SCED^{TM}461) in controlled stirred tank bioreactors.

Different inoculation conditions were firstly evaluated in spinner flasks. The best conditions to promote hESCs attachment were achieved by combining matrigel-coated Cytodex3^{TM} microcarriers (Lock, et al., 2009; Phillips, et al., 2008) in mEF conditioned culture medium supplemented with Rock inhibitor (Figure 5.1A). Additionally, an inoculum concentration of $4.5x10^5$cell/mL was selected to be used in next bioreactor studies as it allowed higher cell yields and a more efficient microcarrier colonization (>90%) during culture time while maintaining constant the levels of SSEA-4 positive cells (Figure 5.1B-E). It is important to highlight that, the long lag phases of 5-7 days presented in all strategies could be a result of cell adaptation to new culture conditions (feeder-free conditions, microcarriers, stirring)

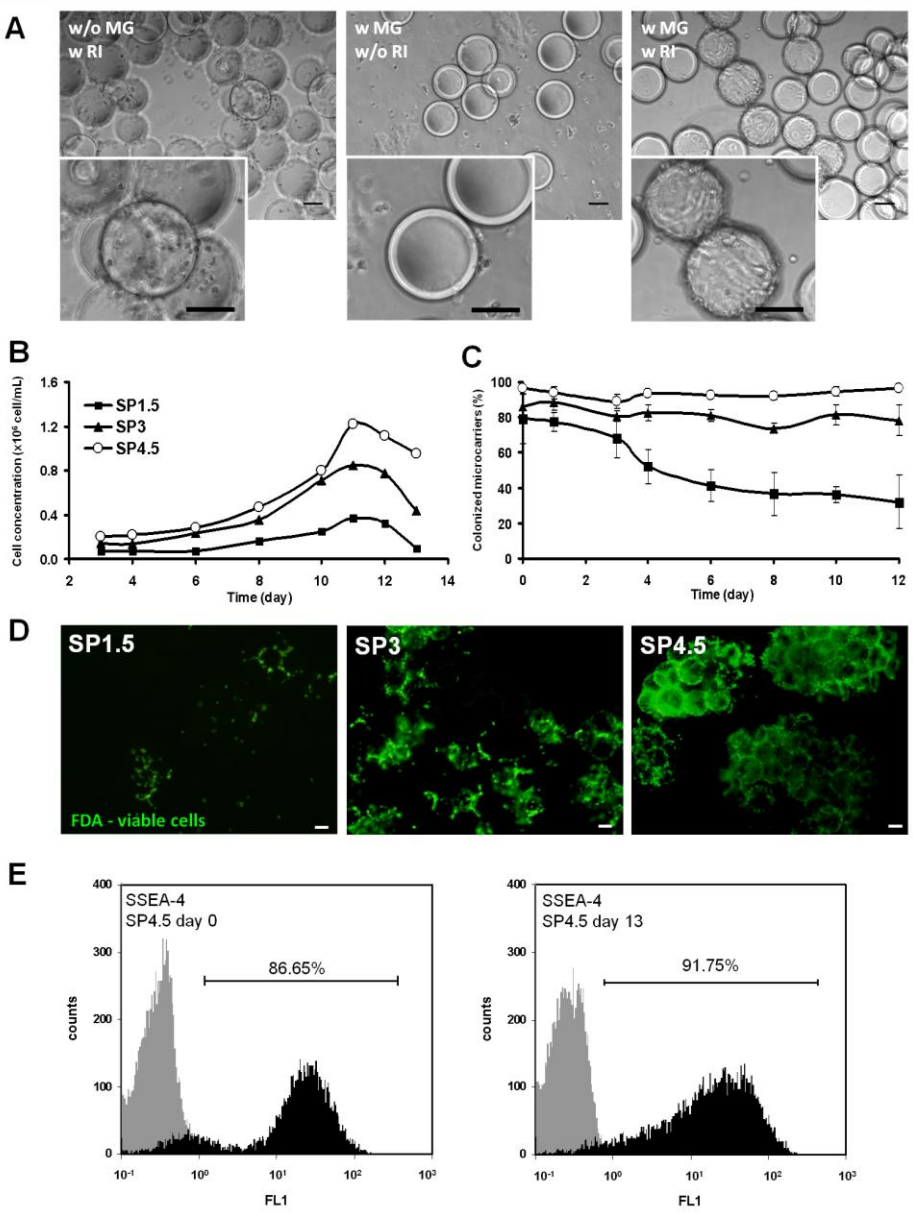


Figure 5.1. Effect of inoculum concentration in the expansion of hESCs adherent to microcarriers. Cells were inoculated at 1.5, 3 and 4.5 x 10^5 cell/mL (SP1.5, SP3 and SP4.5) in spinner flasks. (A) Phase contrast images of hESC cultures 8 hours after inoculation with 4.5 ×10^5 cell/mL in different culture conditions: using Cytodex^{TM}3 microcarriers without (w/o) or with (w) matrigel coating (MG) in the presence (w) or absence (w/o) of Rock Inhibitor (RI). Scale bar: 100 µm. (B) Growth curve expressed in terms of cell number per volume of medium. (C) Profile of microcarrier colonization

expressed in percentage of colonized beads; error bars represent SEM of 3 replicates. (D) Viability analysis of hESC cultures at day 11 stained with fluoresceine diacetate (FDA- live cells, green). Scale bar: 100 µm. (E) Flow cytometry analysis of the expanded cell population in SP4.5: percentage of SSEA-4 positive cells at day 0 and day 13.

The cultivation of hESCs as 3D cell-microcarrier aggregates allowed for higher cell densities than standard static culture (approximately 6.4-fold improvement, Table 5.1). This can be explained by the significantly increased surface area available for cell growth, further facilitating the process scale-up (Fernandes, et al., 2009; Oh, et al., 2009; Kehoe, et al., 2009).

The impact of dissolved oxygen partial pressure (pO_2) upon the expansion of hESCs grown in microcarriers was studied. Two pO_2 values were tested using stirred controlled bioreactors operating in parallel – 5% (BR5%pO_2) and 30% (BR30%pO_2) of air saturation (corresponding to approximately 1% and 6% of oxygen, respectively). Our results demonstrated that BR30%pO_2 improved cell expansion; cell density reached $2.2x10^6$ cell/mL at day 11, in contrast to BR5%pO_2 where expansion was 3 times lower (Figure 5.2A-D, Table 5.1); this lowest pO_2 condition produced a metabolic change to anaerobic metabolism of glucose (Kallos et al., 1999); while similar glucose consumption profiles were observed in both bioreactors, higher lactate production were achieved in BR5%pO_2 (Figure 5.3A,B). In addition oxygen depletion could occur in BR5%pO_2, especially within microcarrier aggregates where the availability of oxygen is limited. The higher energetic metabolism observed in BR30%pO_2 correlated with higher growth rate (Table 5.1), underscoring the importance of using bioreactors with fine control of pO_2 to manipulate hESC metabolism and, consequently, cell growth, without compromising their undifferentiated phenotype (Figure 5.2E).

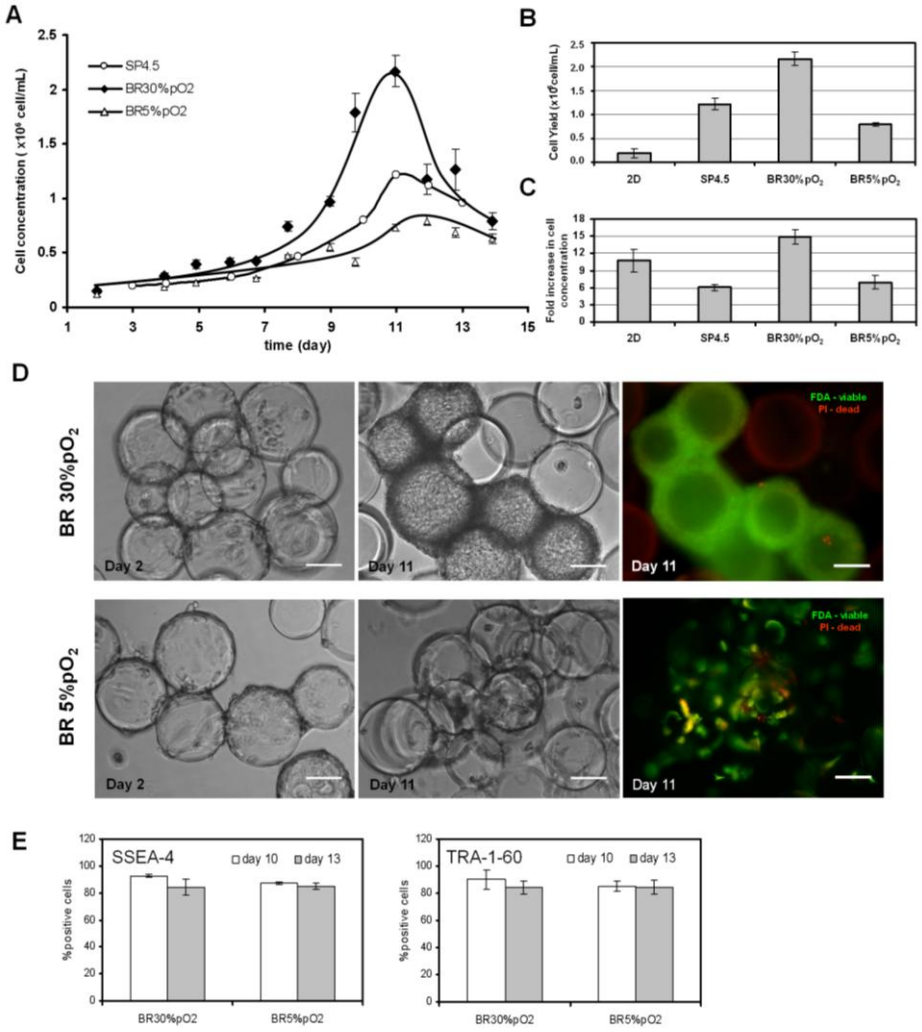


Figure 5.2. Effect of pO_2 in the expansion of hESCs in bioreactors. Cells were inoculated at 4.5 x 10^5 cell/mL and cultured in spinner flasks (SP4.5) or in bioreactors at 30% (BR30%pO_2) and 5% (BR5%pO_2) of pO_2. (A) Growth curve expressed in terms of cell number per volume of medium. (B) Maximum cell yield and (C) fold increase in cell density obtained in static and stirred cultures. (D) Phase contrast and fluorescence images of hESC cultures at day 2 and 11 of both bioreactor experiments. Viability analysis of cultures stained with fluoresceine diacetate (FDA-live cells, green) and propidium iodide (PI- dead cells, red). Scale bar: 100 µm. (E) Flow cytometry analysis of the expanded population: percentage of SSEA-4 and TRA-1-60 positive cells at day 10 and 13 in relation to the inoculum population; error bars represent SD of 2 measurements.

In uncontrolled spinner vessels, placed inside of incubators (where 20% of oxygen is available), the fold increase in cell concentration was 2.5 times lower (Figure 5.2C, Table 5.1), suggesting that the operating conditions of pO_2 were not adequate for optimal hESC expansion. Additionally, the decrease in pH to values that compromised cell viability (pH=6.5), might have contributed to the lower cell yields obtained. In standard 2D systems, where diffusion and availability of oxygen is often limited, it has been demonstrated that physiological levels (1.5-8% oxygen) support self-renewal and reduce spontaneous differentiation (Ezashi, et al., 2005; Prasad, et al., 2009) in contrast to normoxia conditions (20%). Others reported no advantage in the undifferentiated phenotype when hESCs are cultured at 5% of oxygen instead of 20% (Chen et al., 2009). Using pO2-controlled bioreactors, this work contributed to clarify the impact of low oxygen levels upon hESC growth performance.

Moreover, when an automated continuous perfusion system was incorporated in the bioreactor apparatus, hESCs showed an even more efficient energetic metabolism, expressed by higher oxygen consumption and lower lactate production (Figure 5.3B,C), as well as faster cell growth. In this culture, lag phase was reduced and maximum cell density were achieved earlier (day 10) (Figure 5.4A, Table 5.1), confirming that culture performance was enhanced.

When compared to semi-continuous mode, continuous perfusion reduces the fluctuations in the concentration of medium components, such as glucose and lactate (Figure 5.4C-D), which contributes to enhance cell metabolism and growth (Bauwens, et al., 2005; Serra, et al., 2009a). Additionally, the automated strategy assures an efficient operation management, overcoming the main drawbacks of the laborious semi-continuous procedure requiring repeated manipulation.

Table 5.1. Operating parameters and growth kinetics characterization of hESCs expansion using different culture strategies. Cells were inoculated at 4.5 ×10^5 cell/mL in all stirred systems.

Culture Strategy	Static	Spinner vessel	Bioreactor			
	2D monolayers	Cytodex3™ microcarriers	Cytodex3 ™ microcarriers			
pO_2 (% air saturation)	not controlled	not controlled	30%	5%	30%	30%
Operation mode	semi-continuous	semi-continuous	semi-continuous		perfusion	perfusion (+Rap)
Initial cell concentration (×10^5 cell/mL)	0.18± 0.06	2.0± 0.1	1.5± 0.2	1.2± 0.1	1.7± 0.1	2.2± 0.6
Maximum cell density (×10^5 cell/mL)	1.9±0.9	12.2± 0.61	21.7± 1.4	7.9± 0.4	21.1± 3.7	22.6±3.0
Peak in cell density (day)	11	11	11	12	10	10
Fold increase related to initial cell density	10.8± 2.0	6.0± 0.3	14.8± 1.2	6.9± 1.2	12.5± 2.2	10.1± 1.6
Apparent growth rate, μ (day^{-1})	not determined	0.29± 0.01 (R^2=0.99)	0.39± 0.02 (R^2=0.96)	0.13± 0.01 (R^2=0.99)	0.35± 0.04 (R^2=0.98)	0.38± 0.04 (R^2=0.98)
Apparent death rate, K_d (day^{-1})	not determined	0.12± 0.01 (R^2=0.98)	0.33± 0.01 (R^2=0.99)	0.11± 0.01 (R^2=0.95)	0.28± 0.03 (R^2=0.96)	0.07± 0.01 (R^2=0.95)

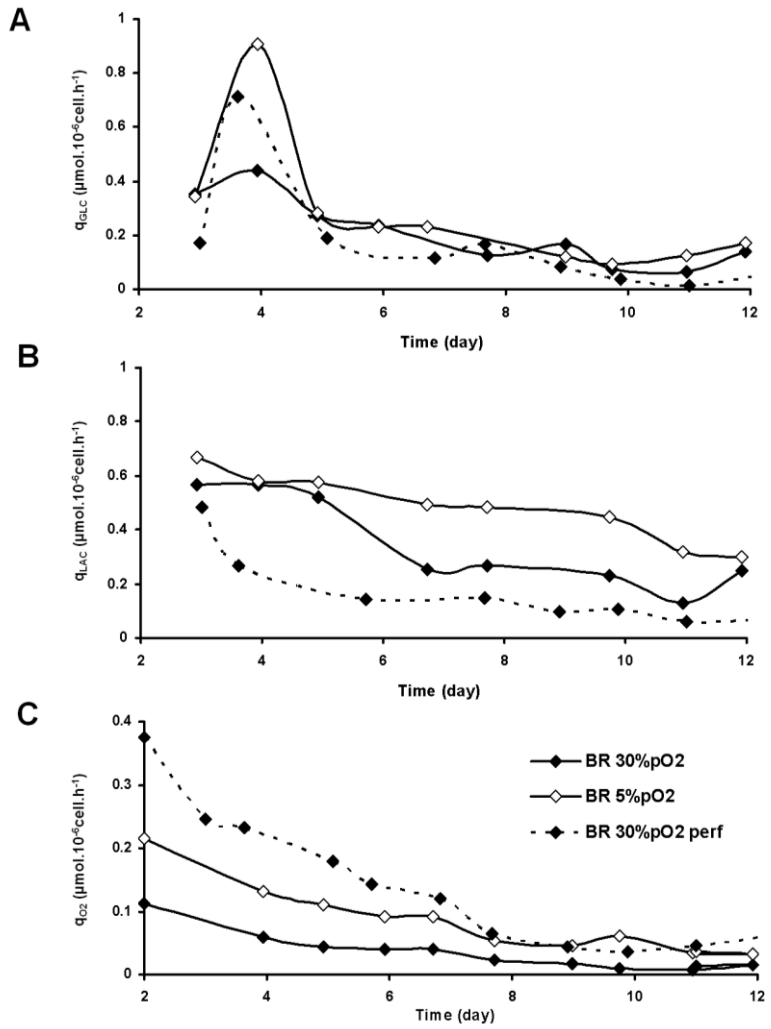


Figure 5.3. Metabolic performance of hESCs cultured in bioreactors. Specific rates of (A) glucose consumption (q_{GLC}), (B) lactate production (q_{LAC}) and (C) oxygen consumption (q_{O2}) of hESCs cultured in bioreactors at 1% (BR5%pO_2) and 6% of pO_2, operating in semi-continuous (BR30%pO_2) or in perfusion mode (BR30%pO_2 per).

Taking advantage of the implemented perfusion system, supplementation with Rapamycin was tested since it has been reported to enhance hESC viability (Krawetz et al., 2009). This effect was validated in bioreactors where higher cell densities were maintained for a longer period (Figure 5.4A); cell viability was improved as lower LDH release and death rate (k_d) values were obtained (Figure 5.4B, Table 5.1).

Importantly, hESCs expanded in bioreactors retained their undifferentiated phenotype and pluripotency, evaluated by immunofluorescence microscopy, flow cytometry, qRT-PCR and detection of alkaline phosphatase activity (Figure 5.5A-C). Moreover, these cells *presented in vitro* and *in vivo* pluripotency (Figure 5.5D-E).

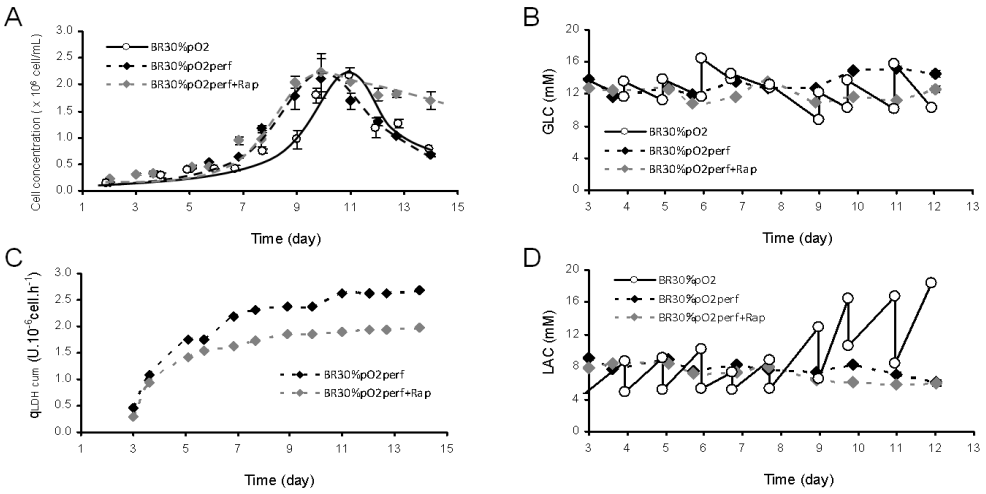
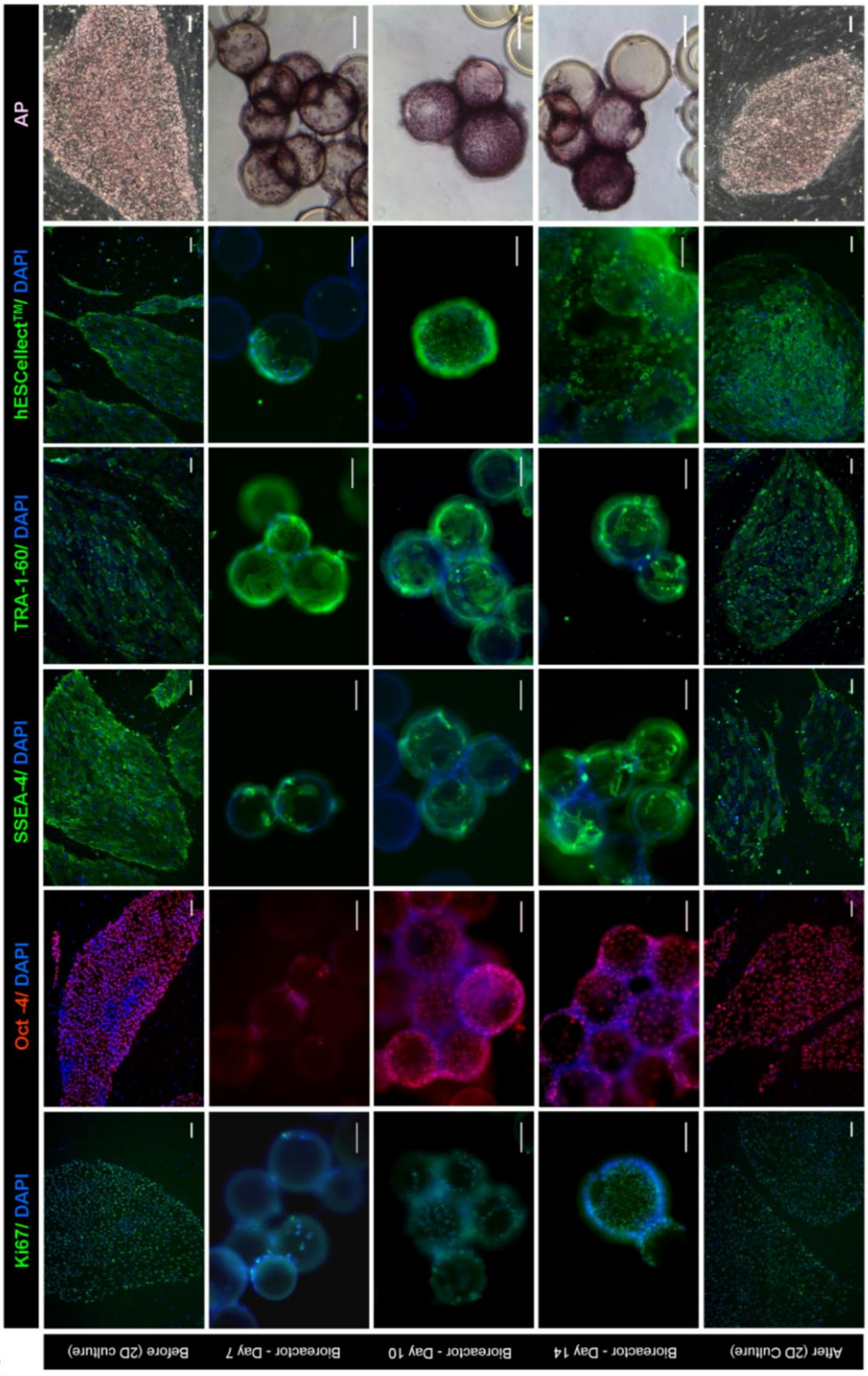


Figure 5.4. Impact of perfusion culture on hESC expansion in bioreactors. Cells were cultured in bioreactors at 30% of pO_2 using different re-feed strategies: semi-continuous (BR30%pO_2) and perfusion with (BR30%pO_2 perf+Rap) or without (BR30%pO_2 perf) Rapamycin supplementation. (A) Growth curves expressed in terms of cell concentration per volume of medium. (B) Cumulative values of specific rates of LDH release during culture time. Error bars denote SD of 2 measurements. (C-D) Profiles of glucose (C) and lactate (D) concentration in culture supernatants.


A
Before (2D culture)
Bioreactor - Day 7
Bioreactor - Day 10
Bioreactor - Day 14
After (2D Culture)
Ki67/ DAPI
Oct -4/ DAPI
SSEA-4/ DAPI
TRA-1-60/ DAPI
hESCellect™/ DAPI
AP

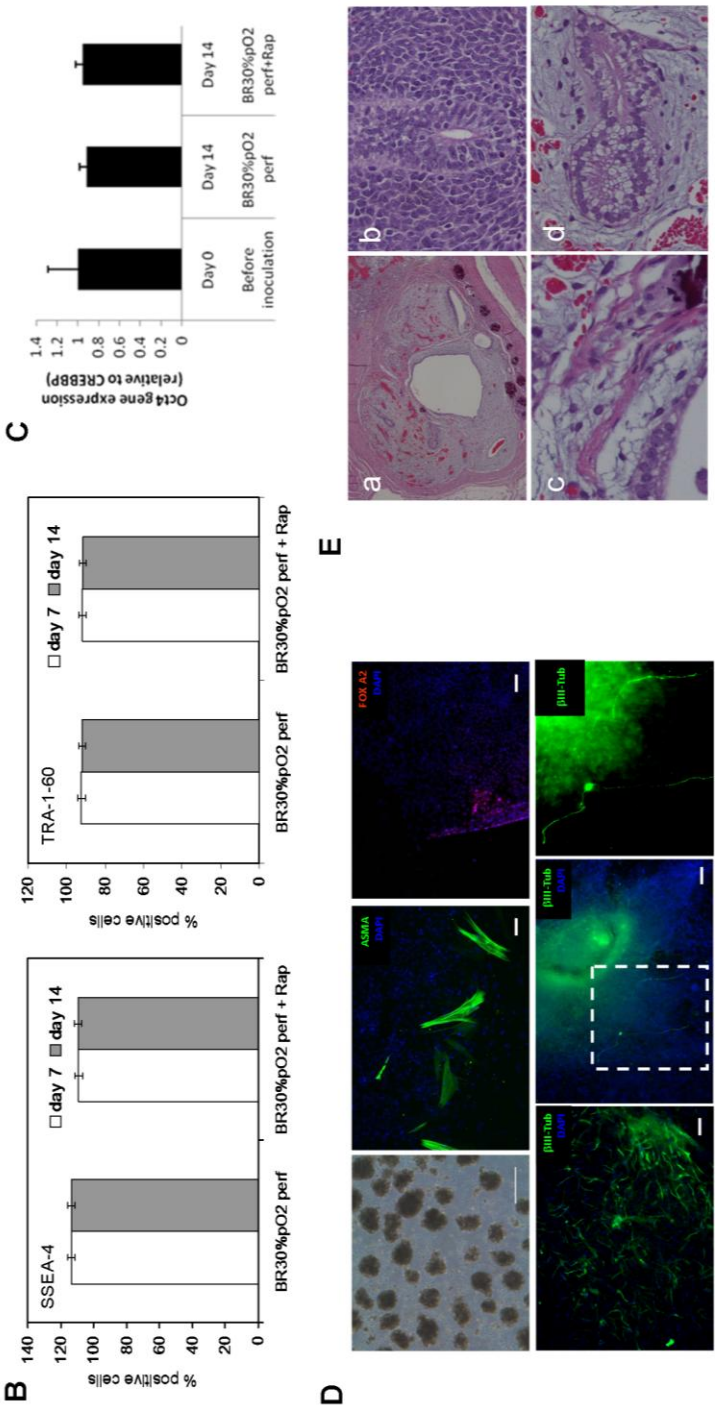

B
SSEA-4
TRA-1-60
% positive cells
day 7
day 14
BR30%pO2 perf
BR30%pO2 perf + Rap
C
Oct4 gene expression (relative to CRE8BP)
Before inoculation
Day 0
Day 14
BR30%pO2 perf
BR30%pO2 perf+Rap
D
ASMA
DAPI
FOX A2
βIII-Tub
E
a
b
c
d

Figure 5.5. Characterization of hESCs expanded in perfused bioreactors. Cells were cultured in bioreactors at 30% of pO_2, operating in continuous perfusion with (BR30%pO_2 perf+Rap) or without (BR30%pO_2 perf) Rapamycin. (A-C) Analysis of hESCs phenotype before inoculation (day 0), during cell growth (days 7, 10 and 14), and after expansion in bioreactors. (A) Immunofluorescence images of ki67, Oct-4, SSEA-4, TRA-1-60, hESCellect^{TM} labeling and phase contrast pictures of alkaline phosphatase (AP) activity staining. Nuclei were labeled with DAPI (blue). Scale bar: 100 µm. (B) Flow cytometry analysis of the expanded population: percentage of SSEA-4 and TRA-1-60 positive cells at days 7 and 14 in relation to the inoculum population; error bars represent SD of 2 measurements. (C) Relative expression levels of oct-4 before (day 0) and after (day 14) expansion. For each sample the expression level was normalized to the CREBBP expression and each sample were normalized to the expression levels at day 0 by using the comparative C_t methods for relative quantification ($\Delta\Delta$ C_t method); error bars represent SD of at least 2 measurements. (D) In vitro pluripotency analysis. hESCs derived from the bioreactor were able to form embryoid bodies (EBs) in non-adherent conditions which differentiated into cells from all three germ layers. Phase contrast micrograph of human embryoid bodies and fluorescence images of differentiated cultures labeled for α–SMA (α smooth muscle actin, mesoderm), FOX-A2 (Forkheadbox A2, endoderm) and βIII-Tub (β tubulin type III, ectoderm). Nuclei were stained with DAPI (blue). Scale bar: 100 µm. (E) hESCs expanded in bioreactors formed teratomas in nude mice. Low magnification photograph showing teratoma on the capsule of testis (a). Typical differentiated tissues form the three germ layers are shown: neuroepithelium (ectoderm) (b), smooth muscle (mesoderm) (c), glandular epithelium and vessels (endoderm) (d).

We are currently applying this technology to other stem cell lines, including human iPS cells, as well for the production of human stem cell derivatives by integrating the differentiation step. It is important to highlight that the bioreactor protocol developed herein could be easily adapted to other culture strategies. Recently, some reports demonstrated the successful expansion of undifferentiated hESC and human iPSC lines as 3D aggregates (Amit et al., 2010; Singh et al., 2010; Steiner et al., 2010), without feeders or microcarriers. In aggregates culture, where the limited diffusion of nutrient/gases within the aggregate could promote spontaneous differentiation, the control of specific bioreactor parameters, including dissolved oxygen, will be critical to preserve the self-renewal ability and further increase the expansion yields.

4. CONCLUSION

Our findings show the importance of controlling pO_2 conditions (30%), achieved in 300mL stirred tank bioreactors, for the efficient production of pluripotent hESCs on microcarries. A 12-fold improvement in the final cell yield was obtained when compared to static 2D cultures, yielding almost 7×10^8 hESCs *per* run. The use of continuous perfusion systems further enhances hESC metabolic performance, ultimately facilitating bioprocess optimization including culture adaptation to xeno-free conditions. The technology developed herein can be translated to clinical and industrial settings, assuring the scalable production of cell-based products in a robust, controlled and automated manner.

5. ACKNOWLEDGMENTS

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CHAPTER 6

MICROENCAPSULATION TECHNOLOGY: A POWERFUL TOOL TO INTEGRATE EXPANSION AND CRYOPRESERVATION OF HUMAN EMBRYONIC STEM CELLS

This chapter was based on the following manuscript:

Serra, M., Correia, C., Malpique, R., Brito, C., Jensen, J., Bjorquist, P., Carrondo, M.J.T., and Alves, P.M. Microencapsulation technology: a powerful tool to integrate expansion and cryopreservation of pluripotent hESCs. PLoS One. accepted

ABSTRACT

The successful implementation of human embryonic stem cells (hESCs)-based technologies requires the production of relevant numbers of well characterized cells and their efficient long-term storage. In this study, cell microencapsulation in alginate was used to develop an integrated bioprocess for expansion and cryopreservation of pluripotent hESCs. Different three-dimensional (3-D) culture strategies were evaluated and compared: microencapsulation of hESCs as single cells, cell aggregates and cells immobilized on microcarriers. Aiming to establish a scalable bioprocess, hESC-microcapsules were cultured in stirred tank bioreactors.

The combination of cell microencapsulation and microcarrier technology resulted in an optimum protocol for the production and storage of pluripotent hESCs. This strategy ensured high expansion ratios (approximately 20-fold increase in cell concentration) and high cell recovery yields after cryopreservation. When compared to non-encapsulated cells, an improvement of up to three-fold in cell survival post-thawing was obtained without compromising hESC characteristics.

Microencapsulation also improved the culture of hESC aggregates by protecting cells from the hydrodynamic shear stress and through aggregate size control, assuring the maintenance of cell pluripotency for up to two weeks.

This work demonstrates, for the first time, that microencapsulation technology is a powerful tool to integrate expansion and cryopreservation of pluripotent hESCs. The 3-D culture strategy developed represents a significant breakthrough towards the translation of hESCs to clinical and industrial applications.

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1. INTRODUCTION

The discoveries on human stem cells, including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), are dynamic and fascinating research fields. The inherent capacity of these cells to grow indefinitely (self-renewal) and to differentiate into all mature cells of the human body (pluripotency) makes them extremely attractive for regenerative medicine, tissue engineering, drug discovery and toxicology (Davila et al., 2004; Jensen et al., 2009; Krtolica et al., 2009; Nirmalanandhan et al., 2009). However, the establishment of effective and robust protocols for large-scale expansion, storage and distribution of hESCs is imperative for the development of high quality therapeutic products or functional screening tools.

The hESCs are routinely cultured in two-dimensional (2-D) systems, namely Petri dishes, well-plates and tissue culture flasks (Ellerstrom et al., 2007). Over the last years, we have been witnessing a constant inadequacy of these 2-D systems in providing the microenvironments that occur in stem cell niches (Lund et al., 2009). Indeed, the inherent variability, lack of environment control and low production yields associated with these culturing approaches are the main drawbacks hampering the development of efficient, scalable and cost-effective stem cell expansion (reviwed in (Placzek et al., 2009)). The low cell recovery yields and the high rates of uncontrolled differentiation obtained after cryopreservation (Heng et al., 2007; Heng et al., 2006; Hunt et al., 2007) also limits their use in clinical or industrial applications.

Many efforts have been put on the development of more efficient hESC culture systems, namely by combining a strategy for 3-D cell organization with a bioreactor-based system where scalability, straightforward operation and homogeneous culture environment are guaranteed (Kehoe et al., 2009; Krawetz et al., 2009; Oh et al., 2009; Serra et al., 2010). Recent studies

show the successful use of stirred tank bioreactors (spinner vessels and environment controlled stirred tank bioreactors) to expand hESCs as aggregates (Dang et al., 2004) or immobilized on microcarriers (Lock et al., 2009; Nie et al., 2009; Oh, et al. 2009; Phillips et al., 2008; Serra, et al. 2010). From a clinical/industrial perspective, these systems still require further improvements in order to increase cell expansion yields and ensure efficient bioprocess integration with cryopreservation protocols. In fact, stirred culture vessels often apply mechanical forces (mixing and eventually perfusion) to the cells, that can ultimately compromise cell viability, morphology, gene expression and differentiation potential (Sargent et al., 2010). The excessive aggregate/microcarrier clumping observed during culture is another concern since it may cause the formation of necrotic centers and/or promote spontaneous differentiation, reducing cell expansion yields. Moreover, the development of effective cryopreservation protocols capable of assuring efficient cell banking after large-scale expansion is still lacking. Although Nie *et al* reported a new method for the cryopreservation of hESCs adherent on microcarriers (Nie, et al. 2009), this protocol needs further optimization in order to remove animal feeder cells and improve cell attachment/survival after thawing.

Cell microencapsulation technology is an attractive approach to overcome these bioprocessing challenges since it provides cell protection from hydrodynamic shear and prevents excessive aggregates agglomeration while allowing efficient diffusion of nutrients, growth factors and gases due to the pore size (Hwang et al., 2009; Zimmermann et al., 2007). Several hydrogels have been used to enhance the culture of hESCs including alginate (Siti-Ismail et al., 2008), poly (lactic-co-glycolic acid)/poly(l-lactic acid) scaffolds (Levenberg et al., 2003) and hydrogels of agarose (Dang, et al. 2004), chitosan (Li et al., 2010) and hyaluronic acid (Gerecht et al., 2007). Alginate is the most common encapsulation material (Chayosumrit

et al., 2010; Jing et al., 2010; Siti-Ismail, et al. 2008) due to its intrinsic properties including biocompatibility, biosafety and permeability (Orive et al., 2003). The production of alginate cell-microcapsules can be performed under safe and physiological conditions (e.g. physiological temperature and pH, use of isotonic solutions instead of cytotoxic solvents) (de Vos et al., 2006) and using good manufacturing practice (GMP) guidelines (Schwinger et al., 2002), conditions that potentiate the use of this technology in cell-based therapies. Indeed, it was already reported that alginate microcapsules have great potential for transplantation of Langerhans' islets and other factor-secreting cells and tissues (Freimark et al., 2010; Zimmermann et al., 2005).

Cell microencapsulation in alginate has been adopted by our group and others to improve the viability and functionality of primary hepatocytes (Miranda et al., 2010; Tostões et al., 2010) and to enhance the differentiation of stem/progenitor cells into different cell types (Bauwens et al., 2005; Delcroix et al., 2010; Goldstein et al., 2001; Kuo et al., 2006; Lee et al., 2009; Liu et al., 2005; Maguire et al., 2007; Maguire et al., 2006; Nieponice et al., 2008; Wang et al., 2009) in bioreactors. In addition, we recently demonstrated that cell encapsulation in alginate is also a valuable strategy to improve cell viability and the integrity of cell monolayers and neuroshperes after freeze/thawing, since cells are protected against mechanical damages during ice crystallization and the risk of disrupting cell-cell and cell-matrix contacts are reduced through immobilization within the hydrogel (Malpique et al., 2009; Malpique et al., 2010). Despite the success in many (stem) cell types, studies describing the microencapsulation of hESCs are rather limited (Chayosumrit, et al. 2010; Jing, et al. 2010; Siti-Ismail, et al. 2008).

In this work, we report for the first time an efficient integrated bioprocess for expansion and cryopreservation of hESCs, using cell microencapsulation in

alginate. Different strategies were evaluated and compared: microencapsulation of single cells, cell aggregates and cells immobilized on microcarriers. The rationale behind the selection of these strategies was based on the fact that each 3-D approach allows different cell-cell and cell-matrix interactions. Microcapsules containing hESCs were cultured in stirred tank bioreactors (spinner vessels) and, after expansion, cryopreserved in cryovials, aiming at developing a scalable and straightforward bioprocess.

2. MATERIAL AND METHODS

2.1. hESCs culture on feeder layer

hESCs (SCED^{TM}461, Cellartis AB, Göteborg, Sweden) were routinely propagated as colonies in static systems (6 well-plates) on a feeder layer of human foreskin fibroblasts (hFF, ATCC collection), inactivated with mitomycin C (Sigma- Aldrich, Steinheim, Germany), in DMEM-KO culture medium (Knockout^{TM}-DMEM supplemented with 20% (v/v) Knockout-Serum Replacement (KO-SR), 1% (v/v) MEM non-essential amino acids (MEM-NEAA), 0.1 mM 2-mercaptoethanol, 2 mM Glutamax, 1% (v/v) Pen/Strep, 0.5% (v/v) Gentamycin (all from Invitrogen, Paisley, UK)) and 10 ng/mL basic fibroblast growth factor bFGF (Neuilly- Sur- Seine, France, Peprotech), as previously described (Ellerstrom, et al. 2007). Every 10-12 days, i.e. when approximately 75-85% of the surface area of the culture well was covered by hESC colonies, the colonies were digested with TrypLE^{TM} Select (Invitrogen, Paisley, UK), for 6- 8 minutes, and the single cell suspension was transferred to fresh inactivated hFF feeders (at splitting ratios between 1:4 and 1:24). Culture medium was replaced with fresh medium every 1–3 days.

2.2. Preparation of mEFs conditioned medium

For the production of conditioned medium (mEF-CM), mouse embryonic fibroblast (mEFs, Millipore, Billerica, MA, USA) were mitotically inactivated and replated on gelatin-coated in T-flasks (Nunc, Roskilde, Denmark) at 5.5×10^4 cell/cm^2 in DMEM-KO medium without bFGF (0.5 mL/cm^2). Briefly, inactivated mEF were cultured at 37ºC with 5% (v/v) CO_2 (in air) and conditioned media were collected daily for a total of 10 days per batch. Before feeding hESC cultures, mEF-CM was filtered and supplemented with 10 ng/mL bFGF and 0.1 nM Rapamycin (Sigma, Steinheim, Germany).

2.3. Microencapsulation of hESCs

Alginate: Ultra Pure MVG alginate (UP MVG NovaMatrix, Pronova Biomedical, Oslo, Norway) was prepared at a concentration of 1.1% (w/v) in 0.9% (w/v) NaCl solution (Chayosumrit, et al. 2010).

Microcapsules formation: Microcapsules were prepared by passing the alginate-cells mixture using 1 mL syringe through an air- jet generator (kindly provided by Fraunhofer-IBMT, Germany), as described in detail elsewhere (Zimmermann et al., 2001), at an air flow rate of 2-3.5 L/min and an air pressure of 1 bar. These encapsulation conditions yielded microcapsules with a diameter of approximately 400-700 µm. For cross- linkage of the UP MVG alginate, a 100 mM $CaCl_2$/10 mM HEPES solution adjusted to pH 7.4 was used. Alginate microcapsules were washed twice with 0.9% (w/v) NaCl solution and once with DMEM-KO medium before being transferred to culture systems.

Alginate microcapsules dissolution: Ca^{2+}-UP MVG alginate was dissolved by incubating the microcapsules with a chelating solution (50 mM EDTA and 10 mM HEPES in PBS) for 5 min at 37ºC (Chayosumrit, et al. 2010).

2.4. Three-dimensional (3-D) hESC cultures

A schematic diagram describing the main steps of the 3-D culture strategies developed herein is outlined in Figure 6.1.

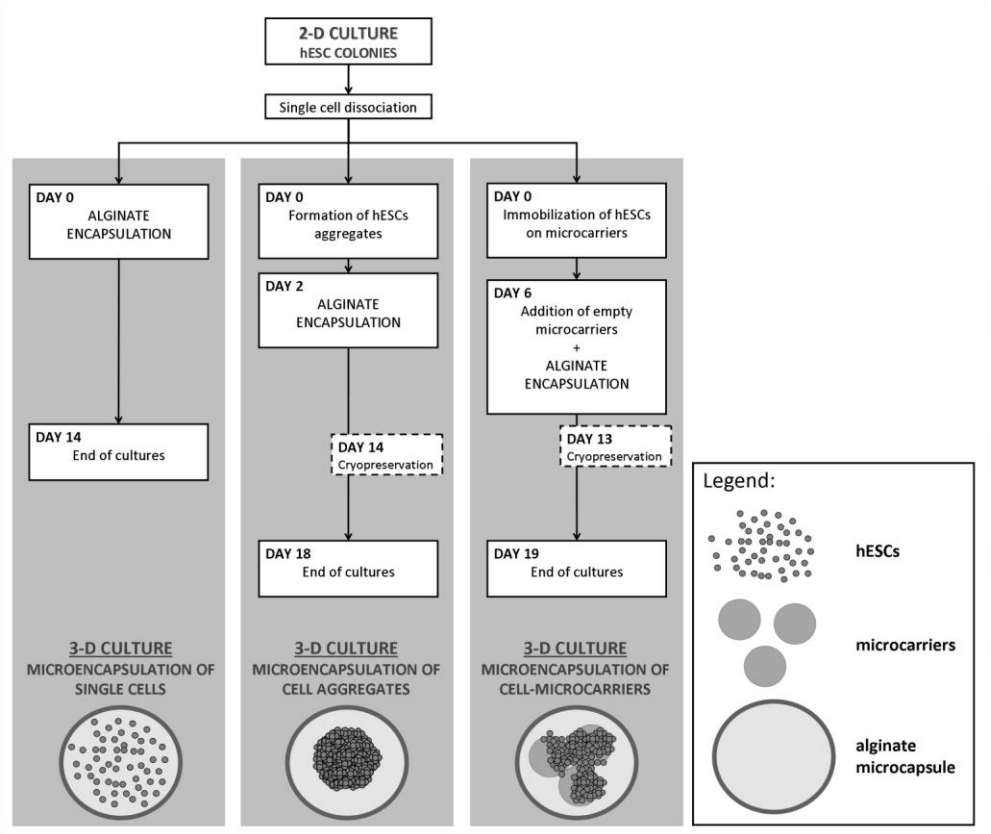


Figure 6.1. Schematic workflow of the main steps of the microencapsulated 3-D culture strategies developed for expansion and cryopreservation of hESCs.

Encapsulation of single cells: Before detachment from 2-D static cultures, hESCs colonies were pre-treated for 1 h with 5 µM Y-27632, a selective Rho kinase (ROCK) inhibitor (ROCKi, Calbiochem Nottingham, UK). The single cell suspension, obtained after colony dissociation with TrypLE Select, was immediately encapsulated at different concentrations in alginate (0.75, 2 and 3 x10^6 cell/mL alginate). hESCs-microcapsules were

then inoculated into 125 mL Erlenmeyer (Corning, Corning, NY, USA) and cultured in 15 mL mEF-CM supplemented with 10 µM ROCKi, at 37ºC and 5% CO_2 in an orbital shaker with an agitation of 70 rpm. In all conditions tested, cells were inoculated at 1.5 $\times 10^5$ cell/mL.

Encapsulation of hESC aggregates: hESCs were dissociated from the 2-D static cultures and inoculated as single cells at 1.5 $\times 10^5$ cell/mL into Erlenmeyer (Corning, Corning, NY, USA). Cells were cultured in 50 mL mEF-CM supplemented with 10 µM ROCKi, at 37ºC and 5% CO_2, using an orbital agitation of 70 rpm. Encapsulation was performed at day 2; aggregates were pre-treated with 5 µM ROCKi for 1 h and then transferred to 15 mL tubes to allow their deposition and culture medium removal. After addition of alginate, aggregates were encapsulated, transferred to 125 mL spinner vessels (Wheaton, Techne, NJ, USA) equipped with paddle impellers and cultured in 100 mL of mEF-CM at 45 rpm for additional 16 days. Culture medium was partially replaced three times a week. This was done by stopping agitation (to induce microcapsules deposition), removing 50% of the medium and feeding with 50% of fresh medium. Cultures of non-encapsulated aggregates were also performed in parallel and used as control. Both cultures were monitored in terms of cell viability, metabolic activity, aggregate size, concentration and composition during time. For flow cytometry analysis, aggregates were transferred to gelatin coated surfaces, in mEF-CM, where cells were able to migrate. After 2-3 days, cells were dissociated using TrypLE Select and processed for flow cytometry analysis using the protocol described below.

Encapsulation of hESCs immobilized on microcarriers: hESCs were inoculated at 4.5 $\times 10^5$ cell/mL into 125 mL spinner vessels with paddle impellers containing Cytodex3^{TM} microcarriers (2 g/L, GE Healthcare, Uppsala, Sweden). The microcarriers were prepared and sterilized according to the manufacture's recommendation and coated with Matrigel

(BD Biosciences, Bedford, MS, USA) as described in the literature (Lock and Tzanakakis 2009). Cells were cultured in 25 mL of mEFs-CM supplemented with 10 µM ROCKi, and the spinner vessels were placed at 37ºC, 5% CO_2 under intermittent stirring. After 6 h, fresh mEFs-CM was added to cultures and agitation rate set to 24 rpm. By day 3, more media was added for a final volume of 100 mL. The encapsulation was performed at day 6; empty microcarriers (1 or 2 g/L) coated with Matrigel were added to the cultures 1 h before encapsulation. Within this period, cultures were treated with 5 µM ROCKi. After encapsulation, hESCs were transferred to spinner vessels and cultured in the same conditions for additional 13 days. Medium was partially (50%) replaced daily. Cultures of non-encapsulated cells-microcarriers were also performed and run in parallel as a control. Both cultures were monitored in terms of cell concentration, viability and culture composition during time.

At the end of the expansion process of both cell aggregates and hESC-microcarriers cultures, microcapsules were dissolved and hESC clumps were dissociated and plated on a top of a monolayer of inactivated hFF for further characterization studies to assess cell pluripotency.

2.5. Cell cryopreservation

Cultures of non-encapsulated and encapsulated hESCs were harvested from the spinner vessels and cryopreserved using the slow freezing rate method (Malpique, et al. 2010). The hESC-microcarriers and hESCs aggregates were collected at day 13 and 14 of culture, respectively (Fig. 1), and all samples were pre-treated with 5 µM ROCKi for 1 hour before being cryopreserved.

Freezing: At the moment of freezing, after deposition of the microcapsules, culture medium was removed and cryopreservation medium (90% KO-SR, 10% (v/v) DMSO (Sigma, Steinheim, Germany), 5 µM ROCKi) was added.

Cell suspensions obtained were then transferred to cryovials (Nunc, Roskilde, Denmark) (1 mL/vial). The cells were allowed to equilibrate in the cryopreservation medium for 20 minutes at 4ºC. Samples were frozen to -80ºC in an isopropanol-based freezing system, ("Mr. Frosty", Nalgene, NY, USA) at a rate of 1ºC per minute, and stored in the gas phase of a liquid nitrogen reservoir until their thawing.

Thawing: Following storage, cells were quickly thawed by placing the cryovials in a 37ºC water bath; a stepwise dilution (1:1, 1:2, 1:4) in mEF-CM was performed immediately after thawing in order to dilute the DMSO while reducing the osmotic shock (Malpique, et al. 2010). Cells-microcapsules were transferred to Petri-dishes and cultured for 9 days in mEF-CM supplemented with 5 µM of ROCKi. Media exchange was performed daily. At day 9, microcapsules were dissolved and hESC clumps were dissociated with TrypLE Select; hESCs were transferred to a monolayer of inactivated hFF and maintained in culture for several passages for post- thaw studies of growth and pluripotency.

Assessment of hESCs survival after thawing: The percentage of hESCs survival/recovery after thawing was determined by calculating the ratio between the number of viable hESCs after cryopreservation and the number of initially frozen viable hESCs, counted using a Fuchs- Rosenthal haemocytometer chamber (Brand, Wertheim, Germany) and the Trypan Blue (Invitrogen, Paisley, UK) exclusion method.

2.6. Evaluation of cell viability

Three methods were used to estimate cell viability.

Cell membrane integrity assay: The qualitative assessment of the cell plasma membrane integrity during culture was done using the enzyme substrate fluorescein diacetate (FDA; Sigma-Aldrich, Steinheim, Germany) and the DNA-dye propidium iodide (PI; Sigma-Aldrich, Steinheim,

Germany) as described in the literature (Lock and Tzanakakis 2009). Briefly, cells/microcapsules were incubated with 20 µg/mL FDA and 10 µg/mL PI in PBS for 2-5 min and then visualized using fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

Trypan Blue exclusion method: The total number of viable cells was determined by counting the colorless cells in a Fuchs- Rosenthal haemocytometer chamber after incubation with Trypan Blue dye (0.1% (v/v) in PBS).

Lactate dehydrogenase (LDH) activity: LDH activity from the culture supernatant was determined by monitoring the rate of oxidation of NADH to NAD^+ coupled with the reduction of pyruvate to lactate at 340 nm. The specific rate of LDH release (q_{LDH}) was calculated for each time interval using the following equation: qLDH= (△LDH)/(△t×△Xv), where △LDH is the change in LDH activity over the time period △t, and △Xv is the average of the total cell number during the same period. The cumulative value q_{LDHcum} was estimated by $q_{LDHcum\ i+1} = q_{LDH\ i} + q_{LDH\ i+1}$.

2.7. Evaluation of metabolic activity

AlamarBlue™ assay**:** hESCs metabolic activity was assessed using the metabolic indicator alamarBlue following the manufacture's recommendation (Paisley, UK, Invitrogen). Briefly, 2 mL of hESC culture were incubated overnight with fresh medium containing 10% (v/v) alamarBlue. Fluorescence was measured in 96-well plates using a microwell plate fluorescence reader (FluoroMax-4, Horiba Jobin Yvon).

2.8. Evaluation of cell growth

Apparent growth rate (µ): µ was estimated using a simple first-order kinetic model for cell expansion: $dX/dt = \mu X$, where t (day) is the culture time and X (cell) is the value of viable cells for a specific t. The value of µ was

estimated using this model applied to the slope of the curves during the exponential phase.

Expansion ratio or fold increase (FI) in cell concentration: FI was evaluated based on the ratio X_{MAX}/X_0, where X_{MAX} is the peak of cell density (cell/mL) and X_0 is the lowest cell density (cell/mL).

2.9. Characterization of hESCs

For all culture samples, microcapsules were dissolved prior to analysis. The undifferentiated status of hESCs was evaluated by analyzing the activity of alkaline phosphatase (AP) and by detecting the expression of specific stem cells markers using immunocytochemistry and flow cytometry analysis.

Alkaline Phosphatase (AP) staining: Cultures were stained using an AP activity detection kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions and observed using an inverted phase contrast microscope (Leica Microsystems GmbH).

Immunocytochemistry: Cultures of hESC were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 20 minutes, permeabilized (only for detection of intracellular marker Oct-4) for 5 minutes in 0.1% (w/v) Triton X- 100 (Sigma- Aldrich, Steinheim, Germany) in PBS and subsequently incubated with primary antibody overnight at 4°C. Cells were washed three times in PBS and then incubated with secondary antibodies during 1 h at room temperature in the dark. After three washing steps with PBS, cell nuclei were counterstained with 4,6- diamidino- 2- phenylindole (DAPI, Sigma- Aldrich, Steinheim, Germany). Cells were visualized using spinning disk confocal (Nikon Eclipse Ti-E, confocal scanner: Yokogawa CSU-x1) and inverted (Leica Microsystems GmbH) fluorescence microscopy. In samples of hESC aggregates, an additional permeabilization step was performed before the addition of primary antibodies; cells were incubated with 0.2% fish skin gelatine and 0.1% TX-100 in PBS for 2 h at room

temperature. Primary antibodies used were: Tra-1-60, and Oct-4 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies used were: goat anti-mouse IgM-AlexaFluor488 and goat anti-mouse IgG-AlexaFluor 488 (all from Invitrogen, Paisley, UK).

Flow cytometry: cell clumps were dissociated with TrypLE Select and the single cell suspension was resuspended in washing buffer (WB) solution (5% (v/v) FBS in PBS). After two washing steps, cells were incubated with primary antibody for 1 h at 4ºC, washed three times in WB and then incubated with the secondary antibody for additional 30 min at 4ºC. After 2 washing steps with WB, cells were analyzed in a CyFlow® space (Partec GmbH, Münster, Germany) instrument as reported elsewhere [16]. Ten thousand events were registered *per* sample. Primary antibodies used were: Tra-1-60, SSEA-4, SSEA-1 and isotype control antibodies (all Santa Cruz Biotechnology, Santa Cruz, CA, USA) and hES-Cellect^{TM} (Cellartis AB, Göteborg, Sweden). Secondary antibodies used were: goat anti-mouse IgM-AlexaFluor488 and goat anti-mouse IgG-AlexaFluor 488 (all from Invitrogen, Paisley, UK).

2.10. *In vitro* pluripotency

The cells' pluripotent potential was evaluated *in vitro* via embryoid body (EB) formation and spontaneous differentiation. hESCs were dissociated, transferred to non-adherent Petri dishes (5 ×10^5 cell/mL) and cultured in suspension for 1 week in DMEM-KO medium without bFGF. EBs formed during this time were harvested and cultured in gelatin- coated plates for further 2 weeks (medium was changed three times a week). Differentiated cells were identified using immunocytochemistry as described above. Primary antibodies used were: α-smooth muscle actin (DAKO, Glostrup, Denmark), Forkhead box A2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β tubulin type III (Chemicon, Temecula, CA, USA). Secondary

antibodies used were: goat anti-mouse IgG-AlexaFluor488 and donkey anti-goat IgG-AlexaFluor594 (all from Invitrogen, Paisley, UK).

3. RESULTS

Results previously reported by our group and others demonstrate that it is possible to expand hESCs as aggregates or immobilized on microcarriers in stirred tank bioreactors [13-18]. Aiming to increase further the cell expansion yields, different 3-D cell microencapsulation strategies were evaluated (Figure 6.1). The most promising strategies were selected to evaluate the impact of microencapsulation on cell cryopreservation, with the final goal to implement an integrated bioprocess for the robust expansion and storage of pluripotent hESCs. In this work, calcium 1.1% (w/v) UP MVG alginate microcapsules were used since previous studies reported that the properties of this matrix fulfill the main requisites (permeability, stability and elasticity) to support an efficient hESCs culture (Chayosumrit, et al. 2010).

3.1. Expansion of microencapsulated hESC as single cells

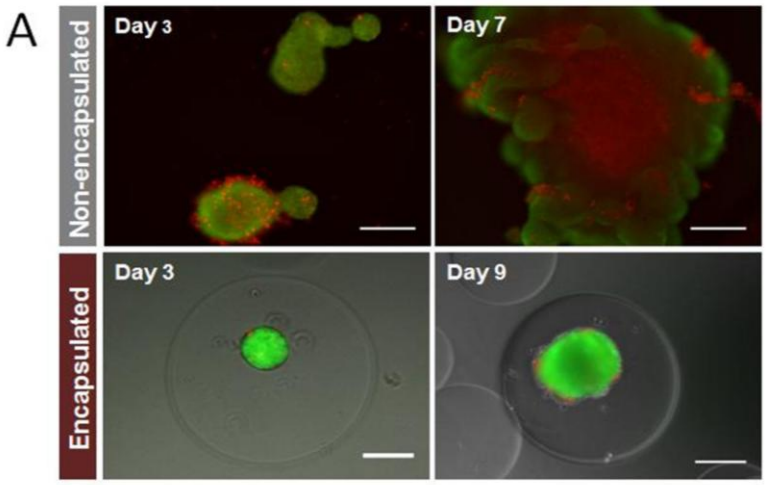
In a first approach we investigated the hypothesis of expanding single hESCs in alginate microcapsules. Cells were encapsulated at different concentrations, 0.75, 2 and 3 $\times 10^6$ cell/mL alginate, and inoculated at 1.5 $\times 10^5$ cell/mL in stirred culture systems. For all conditions tested, cell viability decreased significantly from approximately 95% to 5% after 7 days of cultivation (data not shown). In high cell density microcapsules (3 $\times 10^6$ cell/mL alginate), some cells remained viable, proliferated and formed small clusters, however the percentage of populated microcapsules was very low (<10%, data not shown). These results indicate that the microencapsulation of single cells is not a suitable strategy to expand hESCs.

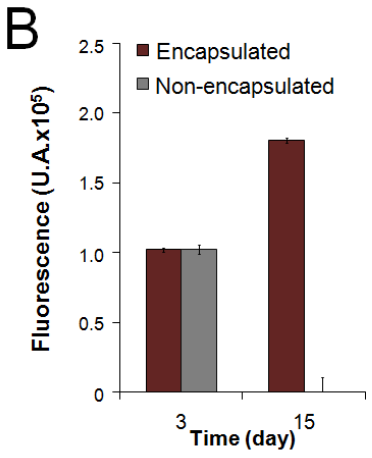
3.2. Expansion of microencapsulated hESC aggregates in stirred tank bioreactors

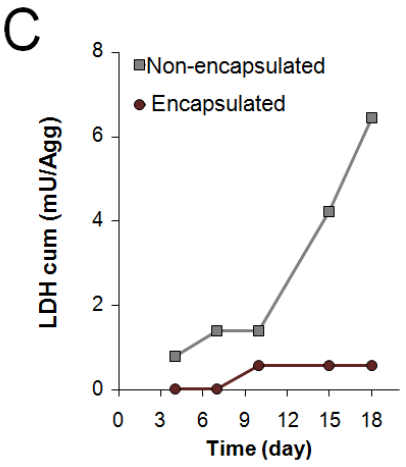
On the second strategy, hESCs were induced to form small cell aggregates after single cell enzymatic dissociation (Figure 6.1). By day 2, aggregates ranging from 30-60 μm were encapsulated to generate approximately 1 aggregate per microcapsule, and transferred to spinner vessels.

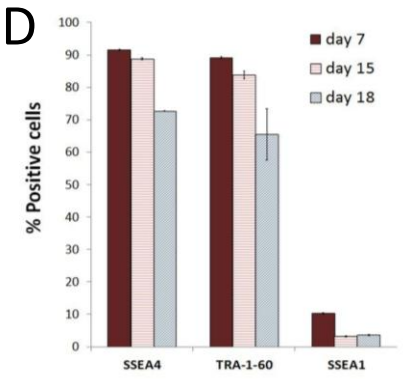
The results show that the microencapsulation of aggregates enhanced the culture performance of hESCs when compared to the microencapsulation of single cells. Aggregates of hESC presented high cell viability and a spherical shape during culture time (Figure 6.2A). After 2 weeks of culture, an increase in aggregate size (5-fold, Table 6.1) and metabolic activity (2-fold, Figure 6.2B) was observed, indicating that hESCs proliferated inside alginate microcapsules. Overall, a significant improvement in cell viability and expansion was obtained when compared to non-encapsulated cultures where aggregates clumped together and formed large (> 1mm in size) irregular structures with necrotic centres (Figure 6.2A). In fact, the pronounced decrease in metabolic activity and the high values of cumulative LDH release confirm that the culture of non-encapsulated hESC aggregates in spinner vessels resulted in an accentuated cell death (Figure 6.2C).

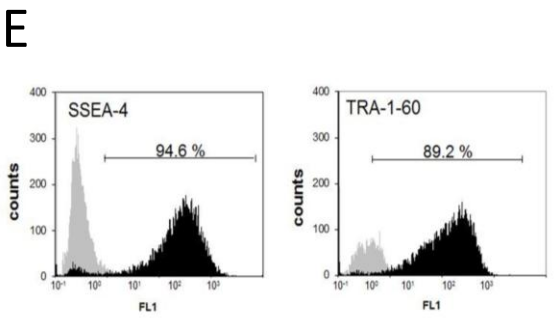
Aggregates collected after microcapsules dissolution maintained their integrity and high cell viability (not shown), thus ensuring an efficient cell characterization. The results show that hESCs expanded as encapsulated 3-D aggregates retained their undifferentiated phenotype during 2 weeks of culture in spinner vessels, as evaluated by immunofluorescence microscopy and flow cytometry (Figure 6.2D-F). By day 7, the percentages of SSEA-4 and TRA-1-60 positive cells were high (94.6% and 89.2%, respectively) indicating that the majority of cells had an undifferentiated character (Figure 6.2D,E).


A
Non-encapsulated
Encapsulated
Day 3
Day 7
Day 3
Day 9


B
Encapsulated
Non-encapsulated
Fluorescence (U.A.x10⁵)
2.5
2.0
1.5
1.0
0.5
0
3
15
Time (day)


C
Non-encapsulated
Encapsulated
LDH cum (mU/Agg)
8
6
4
2
0
0 3 6 9 12 15 18
Time (day)


D
day 7
day 15
day 18
% Positive cells
100
90
80
70
60
50
40
30
20
10
0
SSEA4
TRA-1-60
SSEA1


E
SSEA-4
94.6 %
counts
FL1
TRA-1-60
89.2 %
counts
FL1

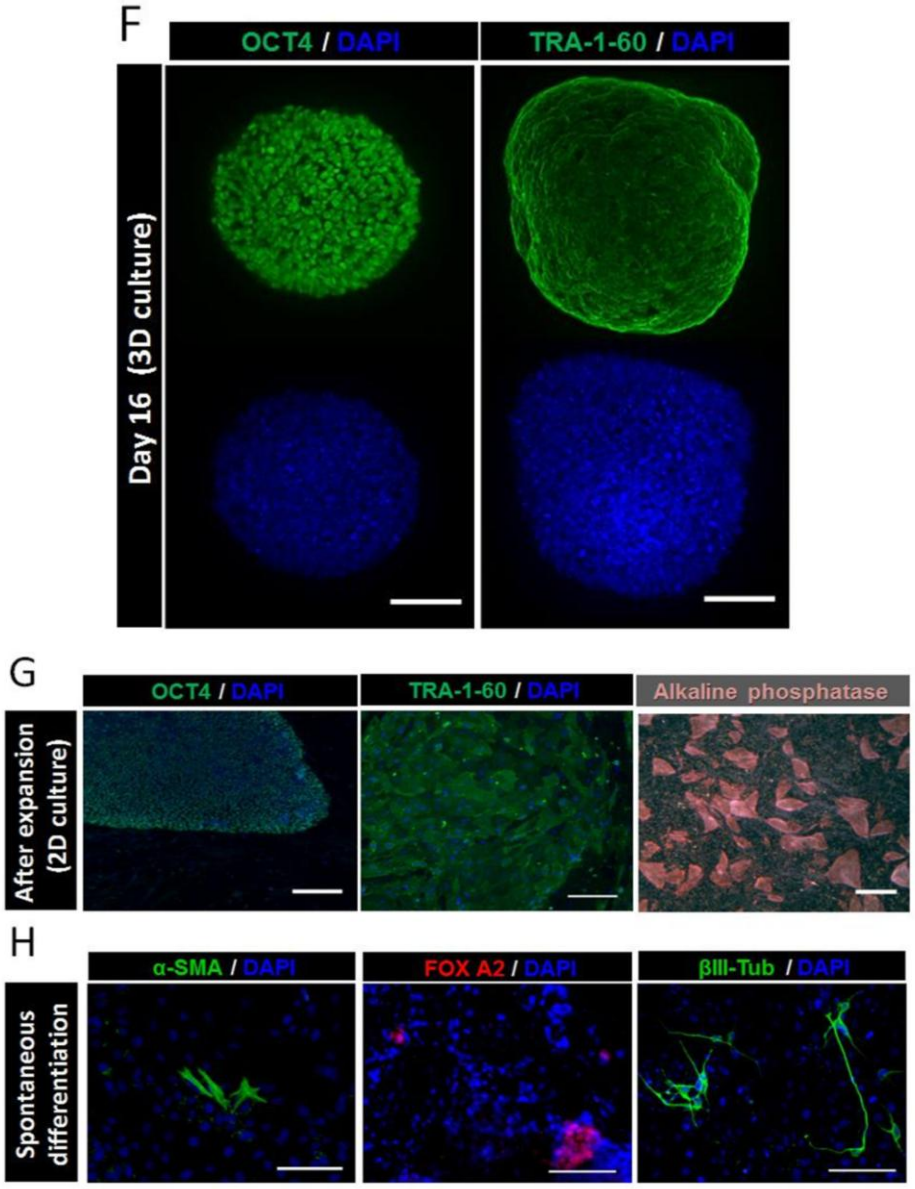


Figure 6.2. Effect of alginate microencapsulation on the expansion of hESC as aggregates. hESC aggregates were encapsulated at day 2 and cultured in spinner vessels. **(A)** Phase contrast and fluorescence images of encapsulated and non-encapsulated cultures at days 3, 7 and 9. Viability of hESC aggregates was assessed by staining with fluoresceine diacetate (FDA-live cells, green) and propidium iodide (PI- dead cells, red). Scale bar: 100 µm. **(B-C)** Cell growth performance of both

encapsulated (purple) and non-encapsulated (grey) cultures. **(B)** Cumulative values of specific rates of LDH release with time. **(C)** Metabolic activity measured by alamarBlue test on the day after microencapsulation (day 3) and at day 15; error bars denote SD of 3 measurements. **(D-H)** Characterization of encapsulated hESC aggregates expanded in spinner vessels. **(D)** Confocal images of aggregates labeled for Oct-4 and TRA-1-60 at day 16 of 3-D culture. Scale bar: 50µm. **(E-F)** Flow cytometry analysis of the expanded population. **(E)** Percentage of SSEA-4, TRA-1-60 and SSEA-1 positive cells at days 7 (purple bars) 14 (pink stripes bars) and 21 (grey stripes bars); error bars represent SD of 2 measurements. **(F)** Histograms obtained in flow cytometry analysis of SSEA-4 and TRA-1-60 positive cells at day 7 of culture. **(G)** Immunofluorescence images of Oct-4 and TRA-1-60 labeling and phase contrast pictures of alkaline phosphatase (AP) activity, staining after expansion (2-D culture). Nuclei were labeled with DAPI (blue). Scale bars: immunofluorescence images - 200 µm, AP image -1mm. **(H)** *In vitro* pluripotency analysis. Microcapsules were dissolved and hESCs were transferred to a monolayer of inactivated hFF. At confluence, colonies were dissociated and hESCs were able to form embryoid bodies (EBs) in non-adherent conditions and differentiated into cells from all three germ layers. Fluorescence images of differentiated cultures labeled for α–SMA (α smooth muscle actin, mesoderm), FOX-A2 (Forkheadbox A2, endoderm) and βIII-Tub (β tubulin type III, ectoderm). Nuclei were stained with DAPI (blue). Scale bar: 100 µm..

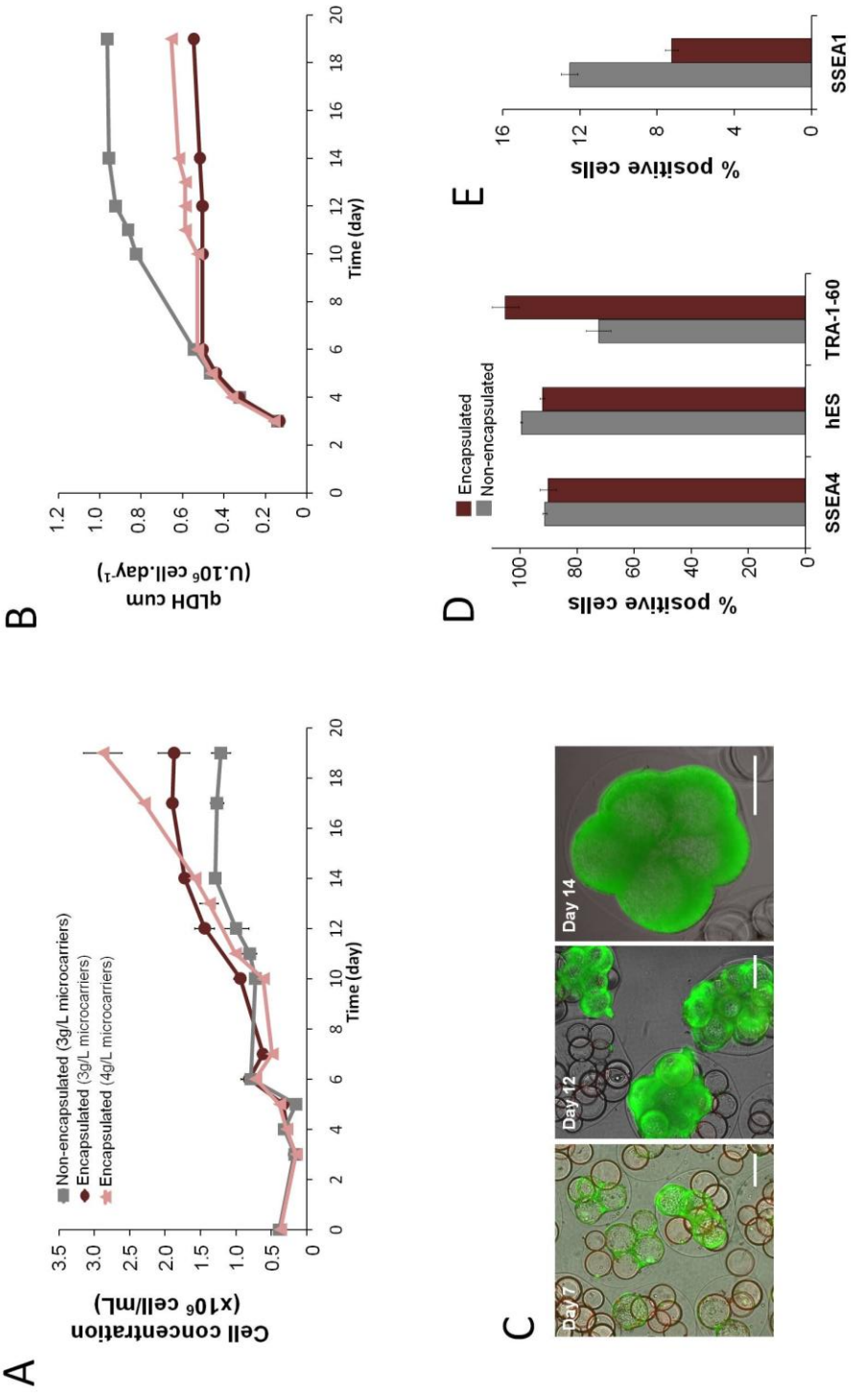
Additionally, in all culture time points the percentages of SSEA-1 positive cells were always below 10% (Figure 6.2D). At day 18, a significant decrease in SSEA-4 and TRA-1-60 positive cells was observed (Figure 6.2D); the presence of EB-like structures (aggregates with irregular shape and cystic cavities) detected at this time point (data not shown), indicates that hESCs were differentiating.

After alginate dissolution, microencapsulated hESC aggregates expanded in the bioreactor were able to form undifferentiated colonies on top of a monolayer of inactivated hFF (Figure 6.2G). Moreover, these cells differentiated spontaneously *in vitro,* via EB formation, into cells from the three germ layers (Figure 6.2H), confirming that they maintained their pluripotent potential.

3.3. Expansion of encapsulated hESC immobilized on microcarriers in stirred tank bioreactors

In the third strategy evaluated, hESCs were immobilized on Matrigel-coated Cytodex 3 microcarriers (3g/L) (Serra, et al. 2010) and encapsulated in alginate. Firstly, the microencapsulation step was tested at different culture time points: 8h (day 0), 1, 3 and 6 days; day 6 was selected since it allowed a higher percentage of microcarriers and microcapsules colonization (data not shown). Preliminary experiments also demonstrated that the addition of empty supports (1g/L) on cell-microcarrier cultures (cells immobilized on microcarriers, 2g/L) immediately before microencapsulation, enhanced further microcapsules colonization and cell expansion yields (data not shown).

Encapsulated hESCs immobilized on microcarriers were cultured for 19 days in spinner vessels (Figure 6.1). The results show that the microencapsulation of cell-microcarriers in alginate markedly enhanced cell viability and expansion when compared to non-encapsulated cultures (Table 6.1, Figure 6.3A,B). By day 19, the fold increase in cell concentration was higher in encapsulated (10.7±0.8%) than in non-encapsulated (7.8±0.3%) cultures, which supports that alginate microcapsules protect the cells from the hydrodynamic shear stress, enhancing cell migration and further proliferation on microcarriers. Moreover, no differences were observed in the apparent growth rates (Table 6.1), indicating that the alginate matrix did not compromise the hESCs proliferation potential.


A
Cell concentration (x10⁶ cell/mL)
Non-encapsulated (3g/L microcarriers)
Encapsulated (3g/L microcarriers)
Encapsulated (4g/L microcarriers)
Time (day)
B
qLDH cum (U.10⁶ cell.day⁻¹)
Time (day)
C
Day 7
Day 12
Day 14
D
% positive cells
Encapsulated
Non-encapsulated
SSEA4
hES
TRA-1-60
E
% positive cells
SSEA1

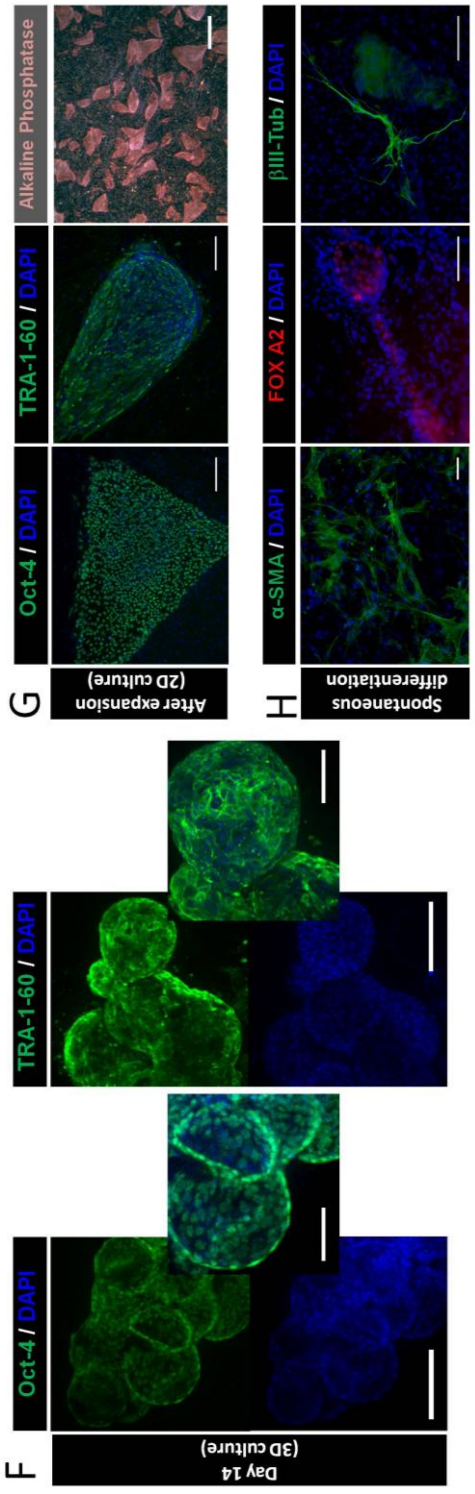


Figure 6.3. Effect of alginate microencapsulation on the expansion of hESCs immobilized on microcarriers. hESCs were immobilized on Matrigel-coated microcarriers (2g/L) and encapsulated at day 6. Before microencapsulation empty coated microcarriers (1g/L and 2g/L) were added. Non-encapsulated (grey) and encapsulated hESCs using 3 g/L (purple) and 4g/L (pink) of microcarriers were cultured in spinner vessels. **(A)** Growth curve expressed in terms of cell number per volume of medium. **(B)** Cumulative values of specific rates of LDH release during culture time. **(C)** Phase contrast and fluorescence images of encapsulated hESC cultures (on 4g/L microcarriers) at days 7, 12 and 14. Viability analysis of cultures stained with fluoresceine diacetate (FDA-live cells, green) and propidium iodide (PI- dead cells, red). Scale bar: 200 µm. **(D-H)** Characterization of encapsulated hESCs immobilized on microcarriers expanded in spinner vessels. **(D)** Flow cytometry analysis of both non-encapsulated (grey bars) and encapsulated (purple bars) hESCs immobilized on microcarriers at the end of the expansion process; percentage of **(D)** SSEA-4, TRA-1-60 and hES-Cellect™ (hES) and **(E)** SSEA-1 positive cells in relation to the 2-D control culture; error bars represent SD of 2 measurements. **(F)** Confocal images of Oct-4 and TRA-1-60 labeling at day 14 of encapsulated 3-D culture. Nuclei were labeled with DAPI (blue). Scale bar: 200 µm, merge images 100 µm. **(G)** Immunofluorescence images of Oct-4 and TRA-1-60 labeling after expansion (2-D culture). Nuclei were labeled with DAPI (blue). Scale bars: 200 µm and 1 mm for immunofluorescence and phase contrast images, respectively. **(H)** *In vitro* pluripotency analysis. Microcapsules were dissolved and hESCs were detached from the microcarriers and transferred to a monolayer of inactivated hFF. At confluence, colonies were dissociated and hESCs were able to form embryoid bodies (EBs) in non-adherent conditions and differentiated into cells from all three germ layers. Fluorescence images of differentiated cultures labeled for α–SMA (α smooth muscle actin, mesoderm), FOX-A2 (Forkheadbox A2, endoderm) and βIII-Tub (β tubulin type III, ectoderm). Nuclei were stained with DAPI (blue). Scale bar: 100 µm.

Table 6.1. Expansion and cryopreservation of encapsulated and non-encapsulated hESC cultures.

Culture Strategy	**hESC aggregates**	
Alginate Microencapsulation	No	Yes
EXPANSION		
Fold increase in metabolic activity (2weeks)	0	2.4±0.2
Initial aggregate size (day 2) (μm)	53±16	53±16
Final aggregate size (day 15) (μm)	-	257±61
CRYOPRESERVATION		
% cell survival	0%	0%

Culture Strategy	**hESCs immobilized on microcarriers**		
Alginate Microencapsulation	No	Yes	Yes
Microcarrier Concentration	3g/L	3g/L	4g/L
EXPANSION			
Initial cell concentration ($\times 10^5$ cell/mL)	1.7±0.3	1.8±0.1	1.5±0.6
Maximum cell concentration ($\times 10^5$ cell/mL)	12.7±0.5	19.0±2.4	28.2±3.8
Expansion ratio/Fold increase related to initial cell concentration	7.7±0.2	10.7±0.8	19.2± 1.8
Apparent growth rate, μ (day^{-1})	0.14± 0.03 (R^2=0.99)	0.15± 0.07 (R^2=0.99)	0.16± 0.02 (R^2=0.94)
CRYOPRESERVATION			
% cell survival:			
Immediately after thawing	53.2±1.1%	100.5±14.0%	-
1 day after thawing	23.8±4.5%	68.8±4.3%	-

Aiming to improve further cell expansion yields, we increased the concentration of microcarriers: 2 g/L of empty supports were added before microencapsulation, yielding a final concentration of 4 g/L. The increase in the surface area available for cell growth contributed to increase the final cell concentration (2.9×10^6 cell/mL corresponding to a 19.2±1.8 of expansion ratio, Table 6.1). Within microcapsules, cells migrated and colonized most of the microcarriers, presenting higher viability during time (Figure 6.3C). It is important to highlight that, using these conditions, the

exponential growth phase was prolonged until day 19 (Figure 6.3A). The culture was aborted at this time point because cells' overgrowth was observed in some microcapsules (data not shown).

After being expanded as encapsulated cell-microcarrier aggregates, hESCs retained their undifferentiated phenotype (Figure 6.3D-F). When compared to non-encapsulated cultures, results were very similar with the exception of TRA-1-60 where higher levels of positive cells were registered in encapsulated cultures (Figure 6.3D). The percentage of SSEA-1 positive cells was higher in non-encapsulated (13.0±0.4%) than in encapsulated cultures (7.8±0.3%) (Figure 6.3E), indicating that, at the end of the expansion process, more cells in an early differentiated state were present in the formers.

Encapsulated cells maintained their capacity to form undifferentiated colonies in 2-D standard monolayer systems (Figure 6.3G) and presented *in vitro* pluripotency; cells were able to form EBs and spontaneously differentiate into cells from the three embryonic germ layers (Figure 6.3H).

3.4. Cryopreservation of hESCs using 3D microencapsulated culture strategies

Since hESCs can be successfully expanded in microcapsules as cell aggregates or adherent to microcarrier surface, we evaluated the possibility of cryopreserving these 3-D structures.. Cells were harvested from the bioreactor cultures at specific culture time points (day 13 and 14 for hESCs-microcarriers and aggregates cultures, respectively) (Figure 6.1) and cryopreserved using a slow rate freezing protocol.

Our results show that alginate microencapsulation did not prevent cell death of cryopreserved hESC aggregates immediately after thawing (Figure 6.4A).

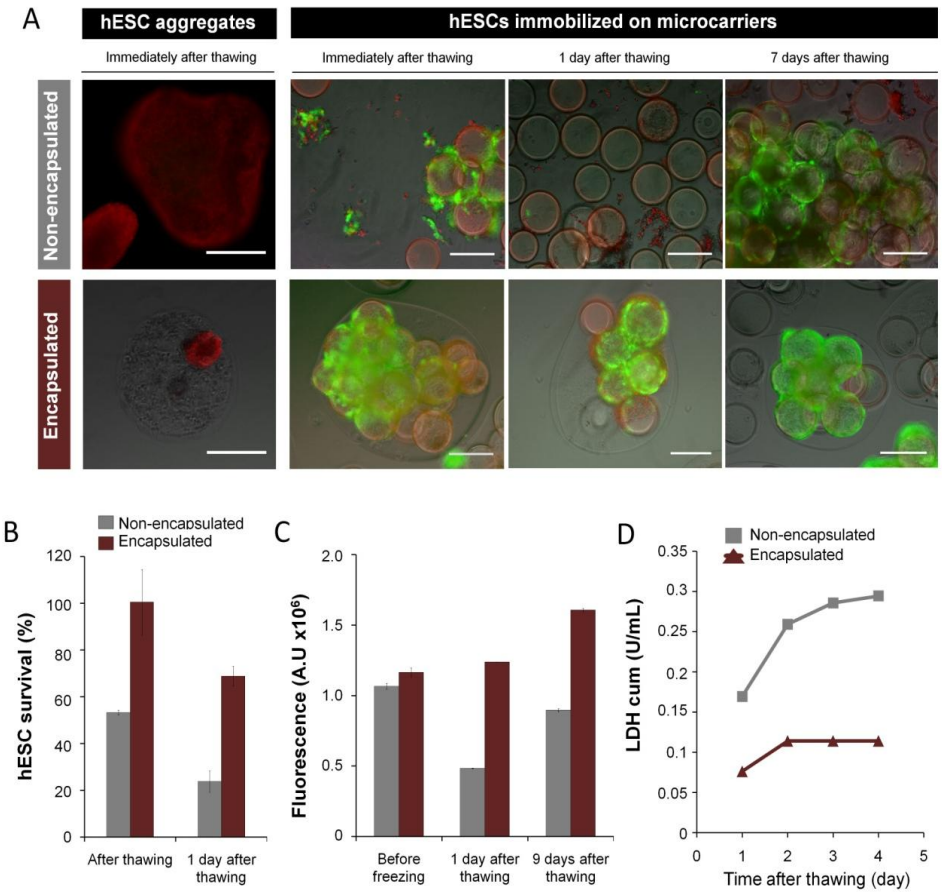


Figure 6.4. Post-thawing survival of non-encapsulated and encapsulated hESCs. Non-encapsulated and encapsulated hESCs were cryopreserved as aggregates or immobilized on microcarrier using slow freeze rate method. (A) Phase contrast and fluorescence images of cryopreserved hESC immediately, 1, 3 and 7 days after thawing. Viability analysis of hESCs stained with fluoresceine diacetate (FDA-live cells, green) and propidium iodide (PI- dead cells, red). Scale bar: 200 μm. (B-G) Post-thawing characterization of non-encapsulated (grey) and encapsulated (purple) hESCs immobilized on microcarriers**.** (B) Percentage of cell survival immediately and one day after thawing; error bars denote SD of 2 measurements. (C) Metabolic activity measured by alamarBlue test before cryopreservation and 1 and 9 days after thawing. Error bars denote SD of 3 measurements. (D) Cumulative values of specific rates of LDH release of cryopreserved hESCs after thawing.

On the contrary, microencapsulated hESCs immobilized on microcarriers presented high cell viability and cell recoveries post-thawing (Figure 6.4A). When compared to non-encapsulated cultures, the results are very promising; immediately and one day after thawing, the percentage of cell survival was higher in encapsulated (day 0=103.7±8.8%, day 1= 71.0±5.0%) than in non-encapsulated cells (day 0= 55.7±4.6%, day 1= 24.9±2.8%) (Figure 6.4B, Table 6.1). Although some cell death occurred in the first days post-thawing, microencapsulated hESCs recovered faster their proliferative and metabolic activity (Figure 6.4C). In non-encapsulated cultures, cells were prone to detach from the microcarriers after thawing resulting in a pronounced cell death (Figure 6.4A); in fact, cells did not reestablish their metabolic activity and the values of LDH were higher than in encapsulated cultures at all time points (Figure 6.4C-D).

To examine if microencapsulated hESCs immobilized on microcarriers maintained their pluripotent characteristics after cryopreservation, cells were characterized 9 days post-thawing and during 5 additional passages on a top of inactivated hFF monolayers. The results confirmed that hESCs maintained their undifferentiated phenotype (Figure 6.5A-C) and the ability to differentiate *in vitro* into cells from the three germ layers (Figure 6.5D).

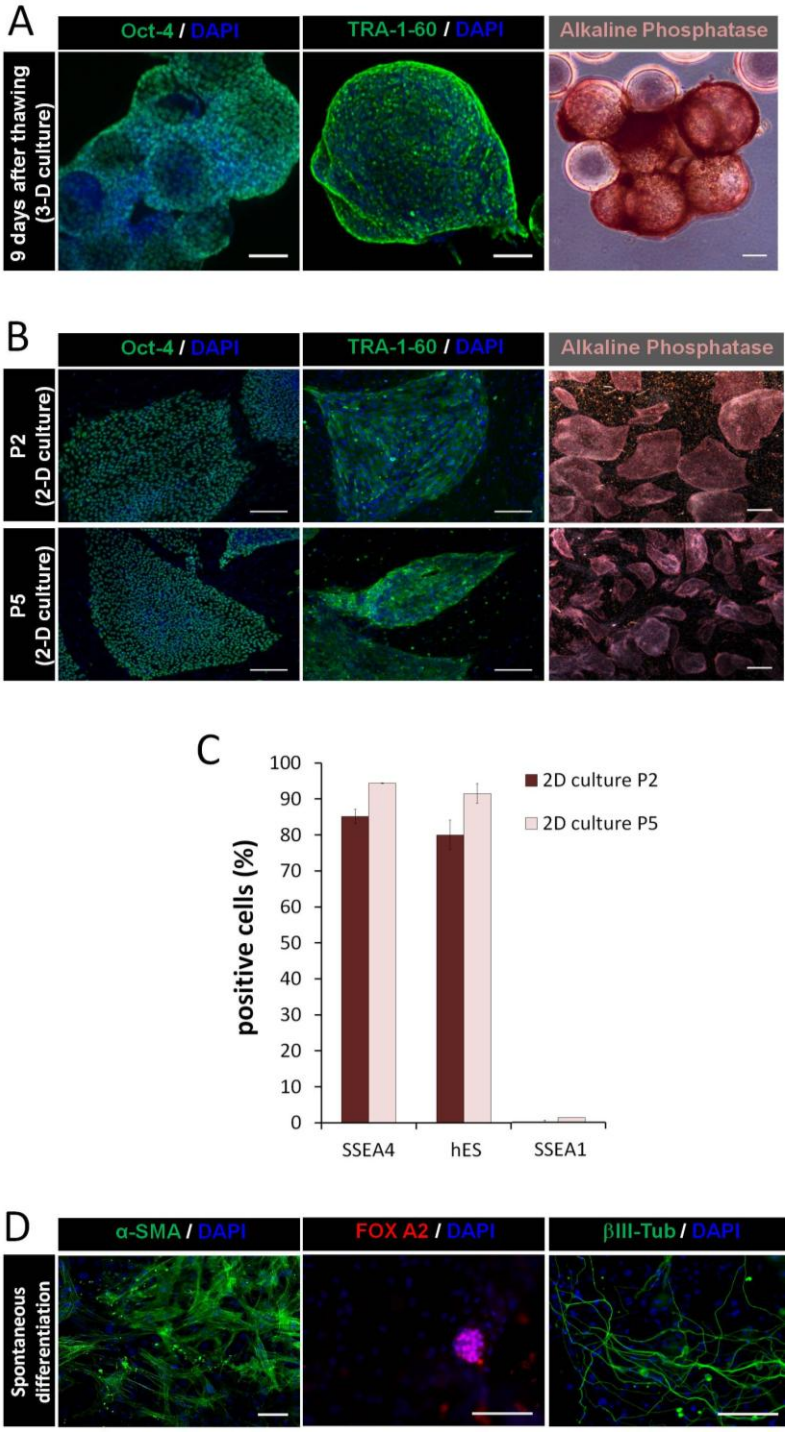

A
Oct-4 / DAPI
TRA-1-60 / DAPI
Alkaline Phosphatase
9 days after thawing (3-D culture)
B
Oct-4 / DAPI
TRA-1-60 / DAPI
Alkaline Phosphatase
P2 (2-D culture)
P5 (2-D culture)
C
positive cells (%)
100
90
80
70
60
50
40
30
20
10
0
2D culture P2
2D culture P5
SSEA4
hES
SSEA1
D
α-SMA / DAPI
FOX A2 / DAPI
βIII-Tub / DAPI
Spontaneous differentiation

Figure 6.5. Post-thawing characterization of encapsulated hESCs immobilized on microcarriers. Phenotype analysis of encapsulated hESC immobilized on Matrigel coated Cytodex3 microcarriers (A) 9 days post-thawing (P0) and (B) after 2 and 5 cell passages in 2-D culture systems (P2 and P5, respectively); confocal images of Oct-4, and TRA-1-60 labeling and phase contrast pictures of alkaline phosphatase (AP) activity. Nuclei were labeled with DAPI (blue). Scale bars: (A) 100 μm and (B) 200 μm for immmunofluorescence images; (A, B) 1mm for phase contrast images. (C) Flow cytometry analysis; percentage of SSEA-4, hES-Cellect^{TM} (hES) and SSEA-1 positive cells after 2 and 5 cell passages post-thawing (P2 and P5, respectively); error bars represent SD of 2 measurements. (D) *In vitro* pluripotency analysis. Microcapsules were dissolved and hESCs were detached from the microcarriers and transferred to a monolayer of inactivated hFF. At confluence, colonies were dissociated and hESCs were able to form embryoid bodies (EBs) in non-adherent conditions and differentiated into cells from all three germ layers. Phase contrast micrograph of human embryoid bodies and fluorescence images of differentiated cultures labeled for α–SMA (α smooth muscle actin, mesoderm), FOX-A2 (Forkheadbox A2, endoderm) and βIII-Tub (β tubulin type III, ectoderm). Nuclei were stained with DAPI (blue). Scale bars: 100 μm.

4. DISCUSSION

Efficient culture strategies are urgently needed to accelerate the transition of hESCs to the clinic and industry. The aim of this study was to develop an integrated bioprocess for expansion and cryopreservation of pluripotent hESCs; our approach consisted in obtaining 3-D culture strategies using cell microencapsulation in alginate. The results obtained show that the combination of cell microencapsulation and microcarrier technology is an optimum protocol for the production and storage of pluripotent hESCs in high quality and relevant quantities.

Cell microencapsulation in alginate proved to be a valuable strategy to improve cell expansion in stirred tank bioreactors, since it ensured a shear stress free microenvironment and avoids excessive clustering of microcarriers or aggregates in culture. This strategy is extremely attractive for use in large-scale bioprocesses, by enabling tighter control of the culture and higher cell expansion yields than non-encapsulated cultures.

Our results show that the microencapsulation of hESCs immobilized on microcarriers is a very efficient system for the long-term culture of undifferentiated stem cells with high cell viability, overcoming the main limitations of both single cells and aggregate cultures. In agreement with other studies confirming that cell-cell and cell-matrix interactions affect significantly stem cell fate decisions (apoptosis, self-renewal, differentiation) (Azarin et al., 2010; Chayosumrit, et al. 2010; Sommar et al., 2010; Wang, et al. 2009), our results show that these interactions cause the improvement of stem cell bioprocesses. In fact, hESCs loose drastically their viability when encapsulated as single cells, even after treatment with Y-27632, a selective ROCK inhibitor known to prevent apoptosis of hESCs after single cell enzymatic dissociation (Chayosumrit, et al. 2010; Watanabe et al., 2007). Moreover, the cultivation of microencapsulated aggregates promotes spontaneous differentiation after 2 weeks of culture. This profile can be explained by the increase in aggregate size (>250 μm), which may limit the diffusion of growth factors and gases within aggregates thereby inducing the formation of EB-like structures and reducing cell proliferation capacity. In a previous study, Siti-Ismai *et al*. reported the long-term feeder-free culture of hESC aggregates in large (approximately 1 mm) calcium alginate capsules, confirming that cells retained their undifferentiated state and pluripotent characteristics for up to 260 days (Siti-Ismail, et al. 2008). This difference in cell behavior may reflect the distinct hESC line and/or culture conditions (alginate matrix, culture medium) used. Nevertheless, the culture of microencapsulated hESC aggregates could be adopted for the production of human stem cell derivatives, by inducing directed differentiation at the second week of culture (when stem cell population is still pluripotent), and bypassed the EB formation step in a controlled manner. There are several studies reporting the use of this strategy to differentiate mouse and/or human ESCs into pancreatic insulin-producing cells (Wang, et al. 2009), hepatocytes

(Maguire, et al. 2007; Maguire, et al. 2006), definitive endoderm (Chayosumrit, et al. 2010), cardiomyocytes (Bauwens, et al. 2005; Jing, et al. 2010) and osteoblasts (Hwang, et al. 2009). High expectations are posed in these culture strategies to potentiate hESCs towards cell therapy and tissue engineering applications (revised in (Murua et al., 2008)).

Another advantage of microcarrier technology in cell expansion processes is the flexibility to adjust easily the area available for cell growth, which further facilitates the process scale-up. From clinical/industrial perspectives, this feature has a tremendous impact in reducing the costs of cell manufacturing by reducing the amount of media, growth factors and other expensive supplements required in stem cell cultivation (Fernandes et al., 2009; Krtolica, et al. 2009). By increasing the concentration of microcarriers we were able to achieve up to $3x10^6$ cell/mL, which corresponds to approximately 15-fold increase in final cell yields when compared to standard 2-D protocols (Serra, et al. 2010). Although performed at small lab scale spinner vessels, the evolved strategies can be easily up-scaled to environment controlled stirred tank bioreactors where scalability, automation and accurate control of culture environment are guaranteed. Indeed, our group has recently demonstrated that the expansion of pluripotent hESCs is improved in stirred tank bioreactors with controlled pO_2 and continuous perfusion (Serra et al., 2010).

This study also demonstrated that the microencapsulation of hESCs immobilized on microcarriers results in an efficient protocol for the cryopreservation of hESCs. Such protocol allows for the recovery of hESCs with high viabilities and undifferentiated levels, and the maintenance of their pluripotent characteristics over several passages in standard culture conditions, enabling their use for further applications. The presence of components of the extracellular matrix on microcarrier cultures (e.g. collagen, laminin) may have contributed to enhance cell survival during

freezing and thawing (Ji et al., 2004; Kim et al., 2004), by reducing post-thaw apoptosis (Heng, et al. 2006; Ji, et al. 2004). In contrast, microencapsulated aggregates showed high cell death immediately after thawing. The limitations in heat and mass (water and cryoprotectant) transfer within aggregates may result in different cryoprotection gradients, possibly leading to cryodamage (Karlsson et al., 1996; Malpique, et al. 2010). In the future, more fundamental studies on the physico-chemical and biophysical phenomena occurring during freezing/thawing of microencapsulated hESC aggregates will allow for a further improvement of this process.

It is important to highlight that, the cryopreservation of hESCs immobilized on microcarriers has already been reported by Nie *et al* (Nie, et al. 2009). The advantage of our strategy is that higher cell recovery yields can be achieved without the use of feeder cells. In fact, the alginate microcapsule allows further improvement of post-thaw cell viability, enhancing cell survival (up to 3-fold) compared to non-encapsulated cultures. Although the underlying mechanisms are still unclear, several studies indicate that maintaining cell-cell contact improves hESC recovery following cryopreservation (Hunt 2007; Ji, et al. 2004). Cell entrapment within alginate microcapsules may help protect hESCs from the adverse effects of cryopreservation not only by preventing the disruption of cell-cell and cell-matrix contacts (Malpique, et al. 2010; Zimmermann, et al. 2005) but also by decreasing exposure to cryoprotectants and preventing the damage caused by intracellular ice formation and propagation (via gap junctions) (Murase et al., 1997; Toner et al., 1993).

To our knowledge this is the first study reporting the successful expansion and cryopreservation of pluripotent hESCs on microcarriers inside alginate microcapsules. More importantly, we describe for the first time an integrated bioprocess which may be used for efficient production, banking

and distribution of hESC in a scalable and straightforward manner. Hopefully, the integrated strategy developed herein will potentiate the translation of hESCs towards a wide range of applications. If hESC can be harvested from the microcapsules they could have immediate use for *in vitro* applications demanding high number of cells, e.g. in high-through-put screening of pharmaceutical compounds. However, from a clinical perspective, further improvements are still required including the adaptation to defined xeno-free culture conditions. The presence of microcarriers within the microcapsules is a major concern, demanding the incorporation of an additional step to release cells from the microcapsules and separate them from the microcarriers before cell transplantation. As an alternative, a biodegradable clinical approved microcarrier could be used. In fact, gelatin and pharmacologically active microcarriers (PAMs) have been used successfully in adult cell therapy for brain neuronal damage and cartilage engineering (revised in (Delcroix, et al. 2010; Hernandez et al., 2010)). Although the type of alginate used in this study has never been tested in clinical studies, it is manufactured in compliance with current GMP and presents low levels of endotoxins (≤100 EU/g), conditions that may boost the use of this matrix in transplantation experiments.

5. CONCLUSION

This study shows that cell microencapsulation in alginate is a powerful tool to integrate expansion and cryopreservation of pluripotent hESCs. Moreover, the combination of cell microencapsulation with microcarrier technology promotes cellular interactions that are essential for the efficient production and storage of hESCs without compromising their viability, self-renewal and pluripotency. The 3-D culture strategy we have developed represents an important step forward in facilitating the translation of hESCs for a broad spectrum of applications in regenerative medicine, tissue

engineering and *in vitro* toxicology. Future studies will include the incorporation of a differentiation step and the development of a fully integrated bioprocess for the expansion, differentiation and storage of hESC derivatives.

6. ACKNOWLEDGMENTS

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CHAPTER 7

DISCUSSION AND CONCLUSIONS

TABLE OF CONTENTS

1. DISCUSSION

Recent advances in stem cell biology and biotechnology have sparked hope that stem cell-based therapies will soon be available to treat devastating maladies such as Parkinson's disease, Type I diabetes and cardiovascular diseases. The successful translation of stem cells to this field requires the development of robust bioprocesses for the production of stem cells and/or their progeny in quantities and qualities that satisfy clinical demands.

The work developed in this thesis aimed at overcoming critical challenges in stem cell expansion and differentiation bioprocesses. Distinct case studies were investigated and novel culture strategies were developed and further optimized. Moreover, several analytical tools were established to monitor stem cell characteristics along culture and also to assess bioprocess yields and end product's purity, quality and quantity. Each presented case faces unique challenges and shows that there is no "optimal/universal" stem cell-based bioprocess capable of embracing all the applications of these cells. Nonetheless, the knowledge gained in the quantitative characterization of expansion and differentiation processes provides important insights for the implementation of, at least, more universal and robust stem cell production platforms.

1.1. Process engineering of stem cells for clinical application

The successful production of stem cell-based products relies on robust bioprocesses that should be designed according to the cell type and characteristics, the needs of the application, and the method requirements (Figure 1.1, page 7).

In this thesis, different culture strategies were developed for three stem cell types, chosen for their unique features and potential applications: adult stem cells isolated from pancreas (rPSC), with the capacity for multilineage differentiation (Chapter 2); human teratocarcinoma stem cells (NT2), which differentiate into neurons after treatment with retinoic acid (Chapters 3 and 4); and pluripotent human embryonic stem cells (hESC) (Chapters 5 and 6). These stem cell types share important characteristics (self-renewal ability and the potential to differentiate into specific cell types) and similar bioprocessing challenges.

An important requirement for the cultivation of these stem cell lines is, in fact, the need for maintaining cell-cell and cell-matrix interactions as well as monitoring specific environmental factors that affect stem cells' fate decisions, in order to better control the culture outcome and boost stem cell bioprocess yields. In this thesis, the development of culturing strategies for 3-D cell organization (cell aggregates, cells immobilized on microcarriers, cell microencapsulation in alginate) combined with the use of bioreactor-based system (where the necessary conditions for cells to guide their fate are "perfectly tuned") demonstrated to be a promising approach to improve stem cell expansion and differentiation yields and to facilitate bioprocess integration.

A schematic representation of the main focus of this thesis is summarized on Figure 7.1. Overall, the following outcomes were achieved:

- Implementation of a robust and scalable protocol for the expansion of undifferentiated rPSCs (Chapter 2) and hESCs (Chapter 5) in environmentally controlled stirred tank bioreactors;
- Development of a robust strategy for the efficient and rapid production of human neurons derived from NT2 cells using stirred tank bioreactors (Chapter 3);

- Development of a scalable and integrated bioprocess for the expansion and neuronal differentiation of NT2 cells (Chapter 4);

- Development of a novel and integrated bioprocess for expansion and cryopreservation of hESCs using cell microencapsulation technology (Chapter 6).

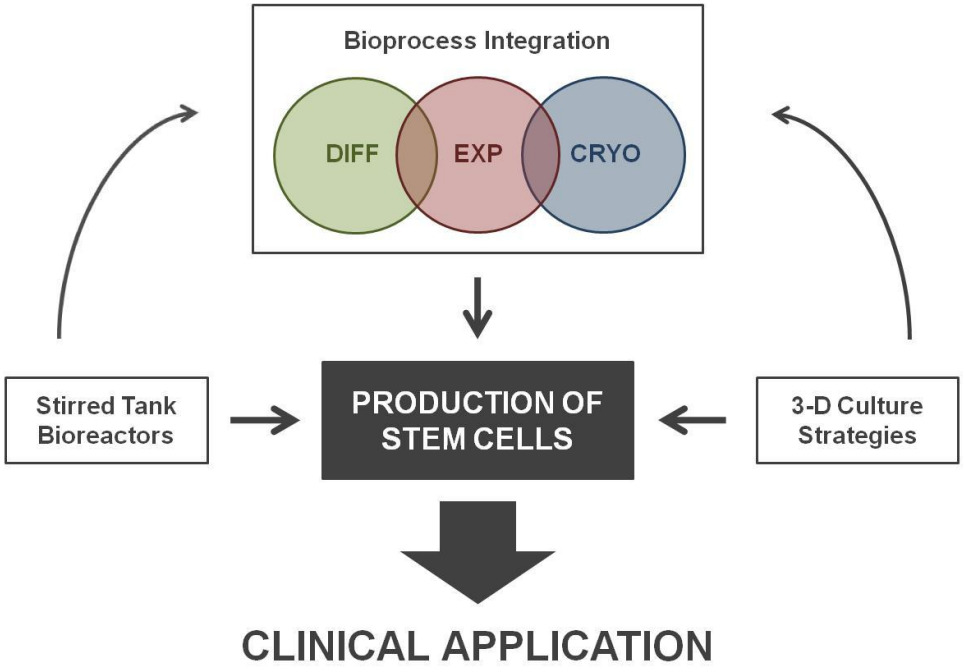

Figure 7.1. Schematic view of the focus and outcomes of the work developed in thesis. (DIFF: differentiation; EXP: Expansion; CRYO: cryopreservation)

These outcomes represent a significant step towards the translation of stem cells from lab scale to clinical trials and larger scales required for industrial applications.

1.1.1. Stirred tank bioreactor: a powerful culture system to potentiate stem cell bioprocessing

An important requirement for the exploitation of stem cells in regenerative medicine is the ability to derive sufficient numbers of cells of a consistent quality in a cost-effective and straightforward manner. Stirred tank bioreactors are an attractive approach for the culture of stem cells. These bioreactors have been heavily utilized in the biotechnology industry for the production of antibodies, enzymes, vaccines and viruses, where nominal volumes of 25 mL to 200L are typically utilized (revised in (Birch et al., 2006; Chu et al., 2001; Kretzmer, 2002)). Hence, stem cell bioprocesses developed in this bioreactor type may be easier to translate to a clinical/industrial production setting than entirely novel designs.

Stirred tank bioreactors are scalable and hydrodynamically well characterized systems with simple design and operation. The main characteristic of these bioreactors is the possibility of culturing cells in a dynamic stirred environment, overcoming the mass and gas transfer limitations of static and other bioreactor systems. Another important feature of these bioreactors is the feasibility to perform non-invasive sampling thus enabling the continuous monitorization/characterization of the stem cell culture status/performance which is critical for process optimization.

In particular, environmentally controlled stirred tank bioreactors allow the on-line monitoring and control of critical culture variables (e.g. temperature, oxygen, pH) known to affect stem cell self-renewal and directed differentiation (revised in Chapter 1). In this thesis, we addressed the importance of controlling the dissolved oxygen and the impact of operation mode to improve the culture of stem cells.

Finally, it is also demonstrated that stirred tank bioreactors can be easily adapted to different 3-D culture strategies (cell aggregates, microcarriers,

microencapsulated cells) and different processes (expansion and differentiation). This versatility makes them appealing “universal culture systems” for use with different stem cell types (including iPS cells) and applications.

1.1.1.1. Control dissolved oxygen to enhance stem cell expansion

All cells of the developing embryo are exposed to oxygen levels *in vivo* (1.5-8%) far below that of atmospheric oxygen levels (approximately 20%), and they differentiate and undergo organogenesis in a low oxygen environment. Therefore, the effects of low oxygen on self-renewal and differentiation of embryonic stem cells have been examined in a number of studies, trying to maximize and control stem cell expansion and/or recapitulate specific differentiation pathways. It has been demonstrating that physiological levels of oxygen support self-renewal of hESCs (Ezashi et al., 2005; Prasad et al., 2009) but also reduce pluripotency gene expression of mESCs (Millman et al., 2009), although some reports are conflicting. Others showed no advantage in the undifferentiated phenotype when hESCs are cultured at 5% of oxygen instead of 20% (Chen et al., 2009). Similar disparities are reported for proliferation rates and differentiation efficiencies. One major problem in the field is the lack of recognition that the oxygen experienced by the cells (pO_{2cell}) is often different from the oxygen in the gas phase (pO_{2gas}), which makes interpretation of the literature difficult. In fact, pO_{2cell} can differ drastically from pO_{2gas}, as it depends on the cell density, culture system (static, stirred, suspension), cellular oxygen consumption rate and oxygen transfer rate in the culture. Knowledge of pO_{2cell} is necessary for interpreting the results of studies and comparing them to data from other studies.

Using environmentally controlled stirred tank bioreactors, the work presented in Chapter 5 contributed to clarify the impact of low pO_{2cell} on hESC growth performance. Although further studies are required to select the best pO_{2cell} condition for maximizing hESC expansion, the results presented in this thesis suggested that 30% of air saturation (which corresponds to approximately 6% of pO_{2cell}) enhances the metabolic performance of hESCs, ultimately improving cell expansion yields without compromising stem cell characteristics such as undifferentiated phenotype and pluripotency. Indeed, more efforts to estimate (Powers et al., 2010) or control pO_{2cell} are essential for rational advance of this field.

1.1.1.2. Culture operation mode to streamline stem cell bioprocessing

Stirred tank bioreactors are highly flexible as they can be handled in different culture operation modes, according to the desired culture outcome. The fed-batch strategy is very suitable for tuning and optimizing cell metabolism (Xie et al., 1994); by providing nutrients in a rational manner, their uptake and consumption are energetically more efficient leading to reduced accumulation of toxic metabolites in culture supernatant (Chapter 4). However, the main disadvantage of this approach is the possibility of depletion of growth factors and/or excessive accumulation of metabolic byproducts and paracrine factors. Thus, perfusion mode was preferentially adopted aiming at improving stem cell bioprocess yields, since it assures the continuous renewal of nutrients and other factors (e.g. Rapamycin) as well as the continuous removal of metabolic byproducts (Chapters 4 and 5).

Within this context, more knowledge regarding the *in vivo* stem cells microenvironment is needed, i.e. take into consideration the existence of concentration gradients in stem cell niches will help to understand their

impact on stem cells' fate decisions. Although not tackled in this thesis, the development of high-throughput technologies combined with the implementation of genomic and proteomic characterization tools will be an important approach to test different identities/combinations/relative levels of nutrients/factors and to analyze the resulting effects on cell phenotype and function (Kirouac et al., 2009; Liu et al., 2009; Bushway et al., 2006). Such combined approach may pave the way for further improvements to the existing expansion strategies, enhancing cellular metabolism by the addition of specific nutrients/aminoacids, or to differentiation processes, by the addition of induced factors. Using stirred tank bioreactors with automated perfusion, the exchange of culture media and/or addition of soluble factors (e.g. growth factors, cytokines and small molecules) may be programmed and performed in a safe operation management, respecting GMP guidelines.

1.1.2. 3-D culture strategies for stem cell bioprocessing: what to choose?

Given the importance of cell-cell and cell-matrix interactions on stem cell fate decisions (apoptosis, self-renewal, differentiation) (Azarin et al., 2010; Chayosumrit et al., 2010; Sommar et al., 2010; Wang et al., 2009) different 3-D culturing strategies were explored in this thesis.

In aggregate cultures, cells can re-establish mutual contacts and specific microenvironments that allow them to express a tissue-like structure, ultimately enhancing cell differentiation and functionality (Burdick et al., 2009; Lund et al., 2009; Pampaloni et al., 2007). Using this 3-D approach, we were able to improve significantly neuronal differentiation process of NT2 cells by increasing the differentiation efficiency and reducing the time needed for differentiation process. At the end, this culturing strategy yields higher amounts of NT2-N neurons with increased purity, as compared with

those routinely obtained using static cultures (Pleasure et al., 1992) (Chapter 3).

Although efficient for expansion and differentiation of NT2 cells, this strategy was unfeasible for the production of undifferentiated rPSCs and hESCs, where cells (and aggregates) clumped together and did not proliferate (Chapters 2 and 6). This profile can be explained by the increase in aggregate size, which may limit the diffusion of nutrients, growth factors and gases within aggregates thereby compromising cell viability and promoting spontaneous differentiation. Further improvements may be considered to better control aggregate size by employing different agitation rates (Cormier et al., 2006; Moreira et al., 1995), using different impeller designs (Singh et al., 2010) and performing repeated enzymatic dissociation steps (Krawetz et al., 2009; zur Nieden et al., 2007).

One method to overcome cellular aggregation in stirred tank bioreactors is to utilize microcarriers to support cell growth. A vast range of microcarriers is currently available (composed by different materials and surface types), in order to allow the culture of different anchorage-dependent cell types in stirred suspension conditions. The selection of the microcarrier type should be performed according to stem cell type (size, morphology, clonal efficiency) and process requirements (expansion, differentiation, cell harvesting). Concerning rPSCs, a more efficient cell adherence and proliferation was obtained using Cytodex 3 microcarriers, which may be explained by the better adhesion of stem cells to the collagen layer that covers the surface of the microcarriers (Chapter 2). However, for the cultivation of hESCs, these microcarriers should be further functionalized with compounds of the extracellular matrix (presented in Matrigel, a complex xenogenic basement membrane matrix) to improve cell attachment and expansion (Chapter 5). The presence of non-defined and animal origin components in the Matrigel matrix, is a major concern for

clinical application of this technology. Future studies should be focus on the development of well defined, GMP compliant and xeno-free matrices for the cultivation of hESCs on microcarriers.

Another advantage of microcarrier technology in cell expansion processes is the flexibility to adjust easily the area available for cell growth, which further facilitates the process scale-up. From clinical/industrial perspectives, this feature has a tremendous impact in reducing the costs of cell manufacturing by reducing the amount of media, growth factors and other expensive supplements required in stem cell cultivation (Fernandes et al., 2009). However, this approach also exhibits some disadvantages, including harmful and unknown effects of shear stress, microcarrier clumping as well as additional operating cost associated to the use microcarriers and the incorporation of additional downstream processes to separate cells from the supports.

Finally, it was also demonstrated herein that cell microencapsulation in alginate is a valuable strategy to improve cell expansion in stirred tank bioreactors, since it ensures a shear stress free microenvironment and avoids excessive clumping of microcarriers or aggregates in culture (Chapter 6). This 3-D strategy is extremely attractive for use in large-scale bioprocesses, enabling tighter control of the culture and higher cell expansion yields than non-encapsulated cultures (improvement of 50%).

In conclusion, the results presented in this thesis show that, so far, there is no optimal 3-D culture strategy capable of embracing all the applications of these cells. Each case faces unique challenges and evaluating them prior processing is crucial to decide on the appropriate method to be used. Nonetheless, it is suggested that cell aggregates strategy should be more appropriated for improving directed differentiation bioprocesses to enhance cell differentiation potential and cell functionality, whereas the use of microcarriers should be more suitable for controlling expansion of

undifferentiated stem cells. The combination of these approaches with cell microencapsulation strategy could be adopted not only to improve further expansion and/or differentiation bioprocesses but also to potentiate hESCs towards stem cell transplantation and tissue engineering applications (revised in (Murua et al., 2008)). The main benefit of cell encapsulation technology is the possibility of designing the scaffold environment with specific biomaterials to create tailored microenvironments that mimic stem cell niches (Burdick et al., 2009; Lund et al., 2009). Thus, the source and properties of the encapsulation material (i.e. elasticity, stability, permeability, biocompatibility and biosafety) should be selected taking into account the culture outcome, the final application and safety issues. In addition, the inoculum cell concentration and the microcarrier type/matrix are important process variables that should also be optimized in the design of 3-D culture approaches to ensure higher stem cell expansion and/or differentiation yields.

It is important to highlight that the cultivation of stem cells in a 3-D approach is not straightforward, requiring exquisite cell culture expertise and the implementation of robust and sensitive characterization tools for 3-D cell culture monitorization, but the resulting bioprocesses and novel stem cell-based applications should more than justify those efforts.

1.1.3. Developing integrated bioprocesses for stem cells: a step forward towards clinical application

The optimal stem cell bioprocess should be able to yield high stem cell production numbers, not by embracing traditional scale-up principles (for example, by the use of large scale bioreactors) but through process intensification, specialization and, more importantly, integration. Specifically, the establishment of systems capable of integrating stem cell inoculation, expansion, differentiation, harvesting and selection would

ultimately result in the scale-up of well differentiated cells to clinically relevant numbers. In fact, when incorporating both expansion and neuronal differentiation steps in an integrated bioprocess, the neuronal differentiation efficiency of NT2 cells was enhanced drastically, while the time needed for differentiation process was reduced; for a differentiation time of 23 days in the bioreactor culture a 10-fold improvement in yield was observed over the static culture protocols lasting 35 days (Chapter 4). This strategy also assures the feasibility of expanding human differentiated neurons derived from a continuous source of pluripotent stem cells. This integrated bioprocess could upstream the application of NT2 cell derived neurons in *in vitro* toxicology, drug screening and cell therapy applications since these cells have been successfully used in neurotoxicity and development neuronal toxicity studies (Hill et al., 2008) as well as in transplantation experiments using mouse models and human stroke patients (Kondziolka et al., 2008).

Another major challenge regarding the application of stem cells in the clinic is the production of stem cell banks of well-characterized cells. Indeed, the development of integrated bioprocesses capable to guarantee efficient cell banking and distribution after large-scale expansion is still lacking. Such protocols must assure high cell survival, low differentiation rates and maintenance of pluripotency post-thawing (Hunt et al. 2007) after expansion and cryopreservation. The results obtained in Chapter 6 show that, cell microencapsulation in alginate is a powerful tool to integrate expansion and cryopreservation of pluripotent hESCs. Moreover, the combination of cell microencapsulation with microcarrier technology promotes cell-cell and cell-matrix interactions that are essential for the efficient production and storage of hESCs without compromising their viability, self-renewal and pluripotency.

One of the main requirements in the development of integrated bioprocesses is to settle an appropriated culture time point for the incorporation of a directed differentiation or cryopreservation step. In both strategies developed in this thesis, cells were differentiated or cryopreserved at the middle of the exponential growth phase since the stem cell population presented high cell viabilities and high percentages of undifferentiated and pluripotent cells.

Although important achievements were obtained in this thesis in what concerns bioprocess integration, further investigations should be performed. Indeed, the development of a fully integrated bioprocess for expansion, differentiation and cryopreservation of stem cell derivatives will clearly support autologous stem cell therapies where is often difficult to predict patient's recovery and availability for injection. Such approach could also be advantageous for the development of clinically compatible iPSC banks for meeting cell therapy needs of the entire world population; the main focus of the "HaploBank project" is to collect samples from haplotypically homozygous donors (for HLA-A, -B and –DR) and implement GMP technology for derivation of iPSC lines, cryopreservation and differentiation into specific therapeutically relevant cell phenotypes.

1.2. Process and product characterization: establishing methods to quantify bioprocess yields and assess product's quality

Due to the lack of accurate, validated methods for quantitative characterization of stem cell expansion and differentiation processes, analytical tools were established for evaluation of bioprocess yield as well as to access end product's quality.

As a starting point, cell growth profiles were evaluated by determining the cell concentration, apparent growth rates and expansion ratios (or fold increase in cell concentration). The analysis of cell viability during

expansion process was performed through qualitative assessment of membrane integrity and apoptosis, and by the quantitative measure of metabolic activity using the alamarBlue assays which, contrary to other probes (e.g. MTT), allows the assessment of the same cell culture repeatedly over a nearly unlimited time period (Chapter 6). The specific consumption rate of nutrients and oxygen and the production of metabolites were also estimated to analyze metabolic performance of the cultures (Chapters 2, 4 and 5).

The development of sensitive assays to detect residual undifferentiated and aberrant stem cell populations is imperative to validate reproducible stem cell bioprocesses. Functional assays should also be implemented to determine the quality and potency of the final cell products. In this thesis, the undifferentiated character of stem cell populations was evaluated by the detection of specific stem cell markers, using a combination of analytical tools (immunofluorescence microscopy, flow cytometry, qRT-PCR). In addition, the self-renewal ability of adult stem cells was confirmed by evaluating telomerase activity (Chapter 2).

Further analyses were carried out to evaluate the differentiation potential of stem cells during and after expansion, including directed differentiation into adipocytes (Chapter 3) or neuronal cells (Chapter 4). Since hESCs can differentiate spontaneously and lose their pluripotent potential when cultivated in 3-D approaches, methods to evaluate the effect of the expansion process on hESC pluripotency were performed (via formation of EBs and teratomas) (chapters 5 and 6). In addition, analysis of cell karyotype should also be carried out to confirm maintenance of normal human chromosome status during long-term culture. These assays are currently being done in collaboration with IPO (Lisbon) as they require exquisite methodologies.

The neuronal differentiation of NT2 cells was monitored by the detection of neuronal markers (Chapters 3 and 4). For a better assessment of neurons/neurosphere functionality, studies on spontaneous electrical activity (Hartley et al., 1999), and synthesis, storage and release of neurotransmitters (Pleasure et al., 1993; Yoshioka et al., 1997; Guillemain et al, 2000) should also be performed. However, due to time limitations these techniques were not pursued further.

In summary, a combination of assays was established to analyze stem cell culture status during expansion and neuronal differentiation. These were designed to allow the assessment of product quality and quantification of process yield. Moreover, the importance of evaluating proliferation, maintenance of cell phenotype and differentiation potential during long-time culture was confirmed, which is a fundamental requirement for further cell-based applications.

The development of novel high-throughput methods allowing for a better characterization of metabolism, of cell genomics and proteomics and understood cell biology is still needed. The insufficient data available in this field strongly compromises and limits the application of worldwide recognized tools for bioprocess description and prediction - mechanistic models - that would be extremely useful for understanding how stem cells respond to specific cues with the ultimate goal of predicting key molecular interactions that impact cell fate (Kirouac et al., 2010).

From an engineer point of view, the development of a fully automated and robust production platform requires the integration of novel technologies to monitor and control not only a set of process parameters (e.g. pH, pO_2, temperature, nutrients, agitation and perfusion rate) but also cell viability, phenotype and functionality throughout the culture process. Therefore, significant benefits would derive from implementing sophisticated sensing and monitoring devices within the manufacturing system. The traceability,

efficacy, safety and quality of the process itself would be highly improved creating defined and robust GMP platforms to deliver safe and efficacious cell therapies.

2. LOOKING AHEAD

The generation of novel and more efficient 3-D culture systems are now more than ever, bringing stem cells to clinic and industry applications. Nonetheless, several hurdles are still delaying a straightforward implementation of these systems.

An important challenge in stem cell bioprocessing is to achieve sufficient numbers of stem cell for clinical applications. In this thesis, the expansion and/or differentiation of stem cells in a 3-D strategy, using stirred tank bioreactors, yielded cell concentrations ranging from 0.2×10^6 cell/mL to 3×10^6 cell/mL (Table 7.1). Therefore, the production of 0.1-10 $x10^9$ cell-based products for personalized cell therapy would require bioreactors with working volumes of few hundred millilitres to a few litres, although issues related to the respective efficiencies of differentiation and downstream process yields (e.g. for purification and selection of a specific cell type, for cell-microcarrier separation) should be considered as well.

Table 7.1. Maximum concentration achieved for each cell-based product investigated in this thesis.

Cell-based product	Maximum cell concentration	
rPSCs	0.2×10^6 cell/mL	(Chapter 2)
NT2 cells	1.8×10^6 cell/mL	(Chapter 4)
NT2-N neurons	0.2×10^6 cell/mL	(Chapter 3)
hESCs	3.0×10^6 cell/mL	(Chapter 6)

From a clinical perspective, further improvements are still required including the adaptation to defined xeno-free culture conditions. There are now well researched chemically defined alternatives available that promise to meet this goal. Another concern is the persistence of undifferentiated stem cells that may form malignant tumors when transplanted in the host (Fujikawa et al., 2005). Therefore, additional efforts will be necessary for the implementation of more efficient methods for differentiation and purification of specialized cells (Brignier et al., 2010), and for the development of a fully integrated bioprocess for the expansion, differentiation, purification and storage of stem cell derivatives.

Finally, the complexity involved in the 3-D cultivation of stem cells in controlled bioreactors requires a multidisciplinary approach. By combining biology, engineering, physics and material sciences, stem cells-based products will be, for sure, more accessible in the near future. Furthermore, the development of mathematical models and biostatistics tools capable to predict the outcome of stem cell bioprocesses (yields of stem cell expansion/differentiation, percentage of cell contaminants, etc.) or to give some insights how end products' quality and purity would impact on the efficacy of stem cell transplantation will be outstanding for the design of novel stem bioprocesses and promising cell-based therapies.

3. CONCLUSIONS

This thesis presents robust and scalable strategies for expansion and differentiation of challenging stem cell-systems, some of each allowing for the establishment of integrated bioprocesses in which cells may be expanded, differentiated or cryopreserved in an efficient and straightforward manner. The results presented herein show that there is no "optimal/universal" stem cell-based bioprocess capable of embracing all the

applications of these cells. Nonetheless, the knowledge gained herein, in which the quantitative characterization of stem cell expansion and differentiation processes is included, provides important insights for the implementation of "more universal" stem cell production platforms, hopefully contributing to potentiate the implementation of novel stem cell-based therapies.

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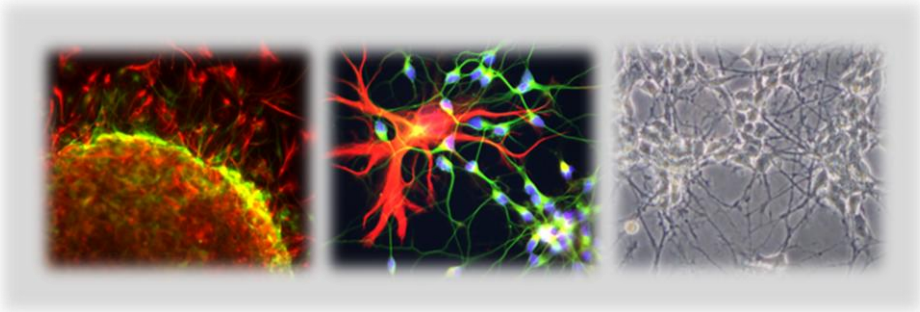
Human stem cells are promising tools for cell replacement therapies and drug screening development. Nonetheless, several hurdles are still delaying a straightforward implementation of these systems.



This thesis intends to explore efficient and scalable bioprocesses for expansion and neuronal differentiation of stem cells in order to ensure the robust production of challenging cell-based products, namely human embryonic stem cells, for a broad spectrum of applications in regenerative medicine, tissue engineering and *in vitro* toxicology.